PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

SCHLICH, G., W.
Mathys & Squire
100 Gray's Inn Road
London WC1X 8AL
ROYAUME-UNI

Date of mailing (day/month/year)
24 February 1999 (24.02.99)

Applicant's or agent's file reference
G2765/38942

IMPORTANT NOTIFICATION

International application No.
PCT/GB98/03001

International filing date (day/month/year)
07 October 1998 (07.10.98)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address
MARLOW, Nicholas, Simon
Reddie & Grose
16 Theobalds Road
London WC1X 8PL
United Kingdom

State of Nationality

State of Residence

Telephone No.
44 171 242 0901

Facsimile No.
44 171 242 3290

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
SCHLICH, G., W.
Mathys & Squire
100 Gray's Inn Road
London WC1X 8AL
United Kingdom

State of Nationality

State of Residence

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+44 171 830 0000

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3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[X] the designated Offices concerned
[ ] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
J. Leitao

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 14 June 1999 (14.06.99)	
International application No. PCT/GB98/03001	**Applicant's or agent's file reference** G2765/38942
International filing date (day/month/year) 07 October 1998 (07.10.98)	**Priority date** (day/month/year) 08 October 1997 (08.10.97)
Applicant DUGGAN, Michael, John et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

28 April 1999 (28.04.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

C. Carrié

Telephone No.: (41-22) 338.83.38

[redacted]TENT COOPERATION TREATY

PCT

09/529130 CK

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
GJL/38942		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 98/03001	07/10/1998	08/10/1997
Applicant		
THE SPEYWOOD LABORATORY LIMITED et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [X] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing

   [ ] filed with the international application.

   [ ] furnished by the applicant separately from the international application,

   [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.

   [ ] Transcribed by this Authority

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. 10

   [X] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/ 03001

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 51-55,60-61
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

[redacted]ational Application No
[redacted]/GB 98/03001

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 33273 A (SPEYWOOD LAB LIMITED ;MICROBIOLOGICAL RES AUTHORITY (GB); FOSTER K) 24 October 1996 cited in the application	1-61
Y	see page 12, line 1 - page 13, line 8; claims see page 13, line 9 - line 13	1-61
P,X	WO 98 07864 A (FOSTER KEITH ALAN ;MICROBIOLOGICAL RES AUTHORITY (GB); QUINN CONRA) 26 February 1998 see claims	1
A	WO 94 28923 A (ALLERGAN INC) 22 December 1994 see claims	

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 January 1999	09/02/1999
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	**Authorized officer** Berte, M

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In. ational Application No
PCT/GB 98/03001

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 18790 A (UNIV MONTANA RES DEV INST ;PASCUAL DAVID (US); BOND CLIFFORD (US);) 29 May 1997 see claims 1,4,5,9,29,30 ---	1-61
A	WO 95 32738 A (ALLERGAN INC ;DOLLY JAMES OLIVER (GB); AOKI KEI ROGER (US); WHEELE) 7 December 1995 ---	
Y	STREIT, WOLFGANG J. ET AL: "Histochemical localization of galactose-containing glycoconjugates in sensory neurons and their processes in the central and peripheral nervous system of the rat" J. HISTOCHEM. CYTOCHEM. (1985), 33(10), 1042-52 CODEN: JHCYAS;ISSN: 0022-1554, 1985, XP002091075 see abstract -----	1-61

INTERNATIONAL SEARCH REPORT

Information on patent family members

In ational Application No
PCT/GB 98/03001

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9633273 A	24-10-1996	AU 5339896 A	07-11-1996
		BG 101984 A	31-07-1998
		CA 2218857 A	24-10-1996
		CZ 9703322 A	18-03-1998
		EP 0826051 A	04-03-1998
		NO 974845 A	18-12-1997
		PL 323006 A	02-03-1998
		SG 52602 A	28-09-1998
WO 9807864 A	26-02-1998	AU 4389597 A	06-03-1998
WO 9428923 A	22-12-1994	AU 683275 B	06-11-1997
		AU 7101894 A	03-01-1995
		EP 0702559 A	27-03-1996
		JP 8511537 T	03-12-1996
WO 9718790 A	29-05-1997	AU 1122697 A	11-06-1997
		CA 2238262 A	29-05-1997
		EP 0869801 A	14-10-1998
WO 9532738 A	07-12-1995	AU 695623 B	20-08-1998
		AU 2622295 A	21-12-1995
		CA 2191754 A	07-12-1995
		EP 0760681 A	12-03-1997
		JP 10500988 T	27-01-1998

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

SCHLICH, George William
MATHYS & SQUIRE
100 Gray's Inn Road
London WC1X 8AL
GRANDE BRETAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	18. 01. 00
Applicant's or agent's file reference: GWS/21094	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB98/03001	07/10/1998	08/10/1997

Applicant
THE SPEYWOOD LABORATORY LIMITED et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

RECEIVED
MATHYS & SQUIRE
21 JAN 2000
REPLY DATE

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	THORNTON, J Tel.+49 89 2399-8072

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
GWS/21094		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB98/03001	07/10/1998	08/10/1997
International Patent Classification (IPC) or national classification and IPC		
A61K47/48		
Applicant		
THE SPEYWOOD LABORATORY LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
28/04/1999	18.01.00
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Giacobbe, S Telephone No. +49 89 2399 8463

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB98/03001

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-24	as originally filed			

Claims, No.:

1-17	as originally filed			
18-57	as received on	16/11/1999	with letter of	15/11/1999

Drawings, sheets:

1/11-11/11	as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-57
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-57
Industrial applicability (IA)	Yes:	Claims	1-50 (cf. also searate sheet for clams 51-57)
	No:	Claims	

2. Citations and explanations

see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

2. Section V

2.1 Cited Documents

The following documents (D) are referred to in this Report:

D1: WO 96 33273 A 24 October 1996

D2: STREIT, WOLFGANG J. ET AL: 'Histochemical localization of galactose-containing glycoconjugates in sensory neurons and their processes in the central and peripheral nervous system of the rat' J. HISTOCHEM. CYTOCHEM. (1985), 33(10), 1042-52

2.2 Art 33(2) PCT (Novelty)

Claims 1-61 fulfill the requirements of Art 33(2) PCT because the available prior art does not disclose agents wherein a specific type of lectins, namely a galactose-binding lectin, is bound to (a derivative of) a clostridial neurotoxin.

2.3 Art 33(3) PCT (Inventive step)

Claims 1-61 do not fulfill the requirements of Art 33(3) PCT.

Document D1, which is considered to represent the most relevant state of the art, discloses (cf. p. 12, l. 1 to p. 15, l. 10 and p. 16, ll. 1, 2) agents for the treatment of pain wherein a clostridial toxin is covalently bound, directly or via a spacer, to a targeting moiety, which can inter alia be a lectin (cf. p. 13, l. 12). The technical problem which the present application sought to solve vis-à-vis this particular prior art document can be formulated as "*how to find a more effective agent for the treatment of pain*". One possible solution is that of finding a targeting molecule which could selectively recognise nociceptive afferent neurones, since then the active molecule, i.e. the clostridium toxin, would be delivered only to these particular neurones. (N.B. Although this is not the only possible approach to the solution of the aforementioned technical problem, it is one which would undoubtedly be considered by the skilled person as possessing a high priority). Document D2 shows (cf. Abstract and p. 1048, 'Discussion') that ' *the peripheral and central nervous system elements with affinity for galactopyranosyl-specific lectins correspond in distribution with neuro- anatomical regions thought to be involved in the transmission and relay of...afferent inputs such as pain and temperature*'. The document further shows that the aforementioned lectins selectively bind to glycoconjugates possessing terminal D-galactose residues localised

on nervous cells situated in the dorsal roots of the spinal chord (cf. p. 1043, 1st column, ll. 1-15: for comparison cf. the present description, Figures 6 and 7), and that these glycoconjugates are associated with cellular membranes and function as recognition molecules (cf. p. 1048, 2nd column, 2nd paragraph of 'Discussion'). The localisation of the conjugates on the cell surface is furthermore confirmed by the fact that they are substrates for the lectins which, as acknowledged by the Applicant (cf. description, p. 7, last sentence), are used as cell surface markers. Document D2 is silent about the susceptibility of these glycoconjugates to internalisation, however this information would not be needed by the skilled person, since he could easily determine this aspect experimentally. Finally, the specific lectins claimed e.g. in claims 8-13 are known from the prior art, as cited by the Applicant (cf. p. 8, ll. 16-31 of the description). From this overall disclosure the skilled person would logically, and without the exercise of any inventive ability, derive that galactopyranosyl-specific lectins specifically recognise the neurons responsible for the transmission of pain, and therefore that they provide the desired solution to the technical problem above.

2.4 Art 33(4) PCT (Industrial applicability)

For the assessment of the present claim 56 on the question of whether it is industrially applicable, no unified criteria exist in the PCT, since the patentability of claims directed to further medical uses is inter alia dependent upon their formulation as well as upon national and regional laws. Furthermore, claims 51-55 and 57, which are directed to a method of treatment of the human or animal body, may not be allowable under some patent systems.

3. Section VI

Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO 98/07864	26.2.98	22.8.97	23.8.96

4. Section VIII

Claims 44 and 45 are redundant since they do not add any essential feature to the claims to which they make reference, but only specify the mechanism of action.

18. An agent according to any preceding Claim in which the lectin has been enzymatically modified.

19. An agent according to any preceding Claim in which the lectin has been chemically modified.

20. An agent according to any preceding Claim wherein, if the heavy chain (H-chain) of a clostridial neurotoxin is present, the H_C domain of the H-chain is removed or modified.

21. An agent according to any preceding claim in which the H-chain, if present, is modified by chemical derivatisation to reduce or remove its native binding affinity for motor neurons.

22. An agent according to any of Claims 1-20 in which the H-chain, if present, is modified by mutation to reduce or remove its native binding affinity for motor neurons.

23. An agent according to any of Claims 1-20 in which the H-chain, if present, is modified by proteolysis.

24. An agent according to Claim 20 in which, if the H-chain is present, the H_C domain is completely removed leaving only the H_N-fragment of a clostridial neurotoxin.

25. An agent according to any preceding Claim in which the derivative of the clostridial neurotoxin, or fragment thereof, is obtained from botulinum neurotoxin.

26. An agent according to any preceding Claim in which the derivative of the clostridial neurotoxin, or fragment thereof, is obtained from botulinum neurotoxin type A.

27. An agent according to Claims 1-25 in which the derivative of the clostridial neurotoxin, or fragment thereof, is obtained from botulinum neurotoxin type B.

28. An agent according to any of Claims 1, 2, or 4-25 (except when dependent on Claim 3) which is formed by the coupling of a galactose-binding lectin to the LH_N fragment of botulinum neurotoxin type A.

29. An agent according to Claim 28 which is formed by the coupling of the galactose-binding lectin from *Erythrina cristagalli* to the LH_N fragment of botulinum neurotoxin type A.

30. An agent according to Claim 28 which is formed by the coupling of the galactose-binding lectin from *Erythrina corallodendron* to the LH_N fragment of botulinum neurotoxin type A.

31. An agent according to Claim 28 which is formed by the coupling of the galactose-binding lectin from *Glycine max* to the LH_N fragment of botulinum neurotoxin type A.

32. An agent according to any preceding Claim in which the H-chain, if present, is obtained from a different clostridial neurotoxin than that from which the L-chain is obtained.

33. An agent according to Claim 32 in which the H-chain is obtained from botulinum neurotoxin type A and the L-chain from botulinum neurotoxin type B.

34. An agent according to Claim 32 in which the H-chain is obtained from botulinum neurotoxin type A and the L-chain from tetanus neurotoxin.

35. An agent according to Claims 33 and 34 in which the H-chain component is the H_N fragment of botulinum neurotoxin type A.

36. An agent according to any preceding Claim in which the L-chain or L-chain fragment is linked to the H-chain, if present, by a direct covalent linkage.

37. An agent according to any of Claims 1-35 in which the L-chain or L-chain fragment is linked to the H-chain, if present, by a covalent linkage which includes one or more spacer regions.

38. An agent according to any preceding Claim in which the clostridial neurotoxin derivative incorporates polypeptides produced by recombinant technology.

39. An agent according to any preceding Claim in which the lectin is linked to the clostridial neurotoxin-derived component by a direct covalent linkage.

40. An agent according to any of Claims 1-38 in which the lectin is linked to the clostridial neurotoxin-derived component by a covalent linkage which includes one or more spacer regions.

41. An agent according to any preceding Claim in which the lectin and clostridial neurotoxin components are produced as a recombinant fusion protein.

42. An agent according to any preceding Claim in which the lectin protein has been modified from its native polypeptide sequence whilst retaining an ability for the protein to bind to oligosaccharide structures, in which the terminal residue is derived from galactose or N-acetylgalactosamine.

43. An agent according to Claim 42 in which the protein modification results from modification of the nucleic acid coding for the lectin protein from its native sequence.

44. An agent according to any preceding Claim which prevents the release of a neurotransmitter or neuromodulator from a primary sensory afferent.

45. An agent according to any preceding Claim which inhibits the release of a neurotransmitter or neuromodulator from a primary nociceptive afferent.

46. A method for obtaining an agent according to any preceding Claim which comprises

the covalent attachment of a galactose-binding lectin to a derivative of a clostridial neurotoxin, in which the derivative of the clostridial neurotoxin comprises the L-chain or an L-chain fragment which includes the active proteolytic domain of the light (L) chain, linked to a molecule or domain with membrane translocating activity.

47. A method for obtaining an agent according to any of Claims 1-45 which comprises the covalent attachment of a galactose-binding lectin to a derivative of a clostridial neurotoxin with the inclusion of one or more spacer regions, in which the derivative of the clostridial neurotoxin comprises the L-chain or an L-chain fragment which includes the active proteolytic enzyme domain of the light (L) chain, linked to a molecule or domain with membrane translocating activity.

48. A method according to Claim 46 or 47 in which the membrane translocation domain is derived from the heavy chain of a clostridial toxin.

49. A method according to Claim 46 or 47 in which the membrane translocation domain is derived from a non-clostridial source.

50. A method for obtaining an agent according to any of Claims 1-45 which comprises constructing a genetic construct which codes for the agent, incorporating said construct into a host organism and expressing the construct to produce the agent.

51. A method of controlling the transmission of sensory information from a primary sensory afferent to a projection neuron by applying the agent of any one of Claims 1-45.

52. A method of controlling the transmission of sensory information from a primary nociceptive afferent to a projection neuron by applying the agent of any one of Claims 1-45.

53. A method according to Claim 51 or Claim 52 wherein the transmission is the release of a neurotransmitter or neuromodulator.

54. A method of controlling the sensation of pain by applying the agent of any one of Claims 1-45.

55. Use of the agent according to any one of Claims 1-45 or a pharmaceutically acceptable salt thereof as a medicament for the alleviation and/or prevention of pain.

56. Use of the agent according to any one of Claims 1-45 in the manufacture of a medicament for the alleviation and/or prevention of pain.

57. A method of alleviating and/or preventing pain which comprises administering an effective dose of the agent according to any one of Claims 1-45.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 47/48

A1

(11) International Publication Number: WO 99/17806

(43) International Publication Date: 15 April 1999 (15.04.99)

(21) International Application Number: PCT/GB98/03001

(22) International Filing Date: 7 October 1998 (07.10.98)

(30) Priority Data:
9721189.0 8 October 1997 (08.10.97) GB

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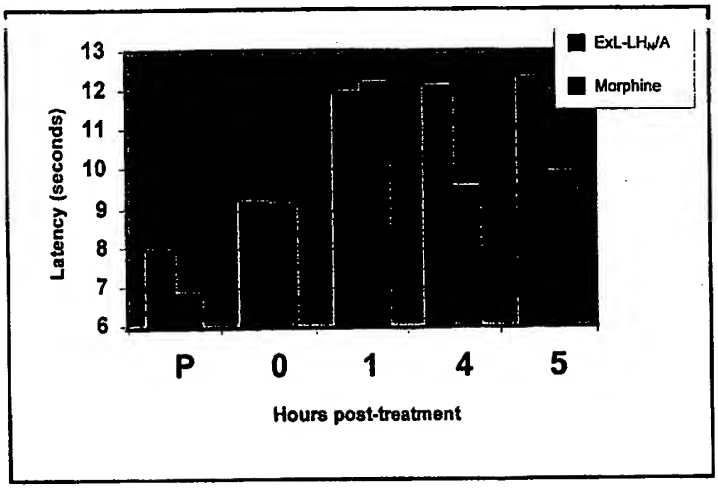

(57) Abstract

A class of novel agents that are able to modify nociceptive afferent function is provided. The agents may inhibit the release of neurotransmitters from discrete populations of neurones and thereby reduce or preferably prevent the transmission of afferent pain signals from peripheral to central pain fibres. They comprise a galastose-binding lectin linked to a derivative of a clostridial neurotoxin. The derivative of the clostridial neurotoxin comprises the L-chain, or a fragment thereof, which includes the active proteolytic enzyme domain of the light (L) chain, linked to a molecule or domain with membrane translocating activity. The agents may be used in or as pharmaceuticals for the treatment of pain, particularly chronic pain.

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Conjugates of galactose-binding lectins and clostridial neurotoxins as analgesics.

Technical field

This invention relates to a class of novel agents that are able to modify nociceptive afferent function. The agents may inhibit the release of neurotransmitters from discrete populations of neurones and thereby reduce or preferably prevent the transmission of afferent pain signals from peripheral to central pain fibres. The agent may be used in or as a pharmaceutical for the treatment of pain, particularly chronic pain.

Background

The sensation of pain due to injury or disease is carried from the periphery to the brain by a multi-neuronal pathway. The first part of this system comprises the primary nociceptive afferents that form synapses with secondary neurones in the dorsal horn of the spinal cord, or the nuclei of the cranial nerves. These synapses pass on the incoming information by the release of neurotransmitters and neuromodulators such as glutamate and substance P. These synapses are, therefore, possible sites for intervention to alleviate pain, indeed one of the modes of action of the opiate analgesics is to down-modulate neurotransmitter release at these synapses.

Unfortunately, the opiates have a number of limitations as drugs. Firstly, there are a number of chronic pain conditions for which the opiates are not effective. Secondly, the opiates have a number of side effects that are mediated both peripherally (constipation) and centrally (respiratory depression and euphoria) which present problems for long term use.

There is, therefore, a need for the development of new pharmaceuticals for the treatment of pain, particularly chronic pain.

One approach to this problem is the use of new agents containing fragments of clostridial neurotoxins (WO96/33273).

The clostridial neurotoxins are proteins with molecular masses of the order of 150 kDa. They are produced by various species of bacterium of the genus *Clostridium*, most importantly *C. tetani* and several strains of *C. botulinum*. There are at present eight different classes of the neurotoxins known: tetanus toxin, and botulinum neurotoxin in its serotypes A, B, C_1, D, E, F and G, and they all share similar structures and modes of action. The clostridial neurotoxins are synthesised by the host bacterium as single polypeptides that are modified post-translationally to form two polypeptide chains joined together by a disulphide bond. The two chains are termed the heavy chain (H), which has a molecular mass of approximately 100 kDa, and the light chain (L), which has a molecular mass of approximately 50 kDa.
Two distinct functions can be identified within the H-chain; binding and translocation. The carboxy-terminal half (H_C) is involved in the high affinity, neurospecific binding of the toxin to cell surface acceptors, whilst the amino-terminal half (H_N) is central to the translocation of the toxin into the neuronal cell. For botulinum neurotoxin type A these domains are considered to reside within amino acid residues 872-1296 for the H_C, amino acid residues 449-871 for the H_N and residues 1-448 for the LC. The minimal domains necessary for the activity of the light chain of clostridial toxins are described in J. Biol. Chem. Vol.267, No.21, July 1992, pages 14721-14729. The eight distinct neurotoxin light chains (L) are highly specific zinc-dependent endopeptidases which each

hydrolyse different but specific peptide bonds in one of three substrate proteins, synaptobrevin, syntaxin or SNAP-25. These substrates are important components of the neurosecretory machinery. The hydrolytic activity of the clostridial toxins results in a prolonged muscular paralysis. The functions of all three identified domains are necessary for the toxic activity of the clostridial endopeptidases.

Some of the clostridial endopeptidases, most notably botulinum neurotoxin type A, have been used as pharmaceutical agents for the treatment of a range of muscle dystonias. The flaccid paralysing action of the native botulinum toxins makes them appropriate for this use.

The use of fragments of clostridial neurotoxins for the desired purpose of analgesia is dependent on the invention of conjugates, or derivatives of these molecules, with a specific binding activity that will deliver the L-chain endopeptidase to the nociceptive afferent neurons in preference to other neurones in the relevant anatomical locus. Delivery of these conjugates includes binding to the cell surface, internalisation via an endosomal compartment and translocation of the clostridial endopeptidase activity into the cytosol.

Targeting of extracellular species to specific intracellular locations following endocytosis involves an appreciation of a number of possible targeting strategies. It is understood that early endosomes are part of the key sorting mechanisms of the cell, routing species to late endosome (and onto lysosomes for degradation), recycling to the cell surface or to the Trans-Golgi Network. Intracellular routing determinants have been suggested that determine the pathway and final destination of

particular species (Mellman, 1996, Annu. Rev. Cell Biol., 12, 575-625).

Current data suggests that translocation of native clostridial neurotoxins occurs from an acidic intracellular compartment, though the exact location and nature of the compartment is unknown (Montecucco & Schiavo, 1994, Mol. Micro. 13, 1-8). In patent WO96/33273 it is proposed that for an agent to be effective, the agent must target to an appropriate compartment for translocation of the toxin. As an example of specific intracellular targeting, internalisation of the NGF-receptor is by specific endocytosis and retrograde routing (initiated by receptor-ligand complex), via acidic endosomes to the cell body, and an agent incorporating NGF is given in support of WO96/33273.

Statement of Invention

The present invention relates to an agent that can reduce and preferably prevent the transmission of pain signals from the periphery to the central nervous system, thereby alleviating the sensation of pain. Specifically, the invention can provide an agent that can reduce and preferably prevent the transmission of pain signals from nociceptive afferents to projection neurones. More specifically, the invention can provide an agent that can inhibit the exocytosis of at least one neurotransmitter or neuromodulator substance from at least one category of nociceptive afferents.

In one aspect of the invention, an agent is provided which can be administered to the spinal cord, and which can inhibit the release of at least one neurotransmitter or neuromodulator from the synaptic terminals of nociceptive afferents terminating in that region of the spinal cord.

In a second aspect of the invention, there is provided an agent which can specifically target defined populations of afferent neurones, so that the effect of the agent is limited to that cell type.

In a third aspect of the invention, there is provided a method of treatment of pain that comprises administering an effective dose of the agent according to the invention.

In a fourth aspect of the invention, the agent can be expressed recombinantly as a fusion protein that includes the required components of the agent.

Definitions

Without wishing to be limited by the definitions set down, it is intended in this description that the following terms have the following meanings:

Light chain means the smaller of the two polypeptide components of any of the clostridial neurotoxins. It is commonly referred to as the L-chain or simply L. An L-chain has a molecular mass of approximately 50 kDa, and it is a metalloprotease exhibiting high substrate specificity for vesicle and/or plasma membrane associated proteins involved in the exocytotic process.

Heavy chain means the larger of the two polypeptide components of any of the clostridial neurotoxins. It is commonly referred to as H-chain or simply H and has a molecular mass of approximately 100 kDa.

H_c fragment means a peptide derived from the H-chain of a clostridial neurotoxin which is responsible for binding of the native holotoxin to cell surface acceptor(s) involved in the intoxicating action of clostridial toxin prior to

internalisation of the toxin into the cell. It may be approximately equivalent to the carboxy-terminal half of the H-chain, or the domain corresponding to that fragment in the intact H-chain.

H_N fragment means a fragment derived from the H-chain of a clostridial neurotoxin approximately equivalent to the amino-terminal half of the H-chain, or the domain corresponding to that fragment in the intact H-chain. It is characterised as:

A portion of the H-chain which enables translocation of that portion of the neurotoxin molecule such that a functional expression of light chain activity occurs within a target cell.

The domain responsible for translocation of the endopeptidase activity, following binding of neurotoxin to its specific cell surface receptor via the binding domain, into the target cell.

The domain responsible for formation of ion-permeable pores in lipid membranes under conditions of low pH.

The domain responsible for increasing the solubility of the entire polypeptide compared to the solubility of light chain alone.

LH_N means a fragment derived from a clostridial neurotoxin that contains the L-chain, or a functional fragment thereof, coupled to a H_N fragment.

BoNT/A means botulinum neurotoxin serotype A, and is a neurotoxin produced by *Clostridium botulinum*; it has a molecular mass of approximately 150kDa.

LH_N/A is LH_N that is derived from *Clostridium botulinum* neurotoxin type A.

Targeting Moiety (TM) means any chemical structure of an agent which functionally interacts with a binding site

causing a physical association between the agent and the surface of a primary sensory afferent.

Primary sensory afferent is a nerve cell that can carry sensory information from the periphery towards the central nervous system.

Primary nociceptive afferent is a nerve cell that can carry sensory information from the periphery towards the central nervous system, where that information can result in a sensation of pain.

Lectin is any protein that binds to oligosaccharide structures.

Galactose-binding lectin is a lectin that binds to oligosaccharide structures in which the terminal residue is derived from galactose or N-acetylgalactosamine.

Detailed Description of the Invention

It can be seen from this disclosure that an agent for reducing or preventing the transmission of pain signals from peripheral, nociceptive afferent neurones to projection neurones has many potential applications in the reduction of the sensation of pain, particularly of severe chronic pain.

Lectins are a class of proteins, often glycoproteins, that bind to carbohydrate structures. Lectins are found across the whole range of life forms from viruses to mammals. The most commonly exploited sources are the abundant lectins found in the seeds of plants. Lectins have previously been labelled and used as cell surface markers.

According to the invention, there is provided an agent that can inhibit the release of at least one neurotransmitter or neuromodulator or both from the synaptic terminals of nociceptive afferents.

It is known that such an agent can be produced based on the use of fragments of clostridial neurotoxin conjugated to a targeting ligand (WO96/33273). Given the known complexity of intracellular transport and the constraints on construct requirements, it is surprising that conjugates between toxin fragments and a specific sub-class of lectins that bind only to galactosyl residues form agents to produce analgesics that are particularly potent and selective. Inventions incorporating such lectins are the subject of this disclosure and several examples are provided.

One example of a class of plant-derived, galactose-binding lectins are those that can be purified from the seeds of the genus *Erythrina*. These lectins have been characterised to exist predominantly as non-covalent dimeric proteins with total molecular weights of approximately 60 kDa. Lectins have been isolated from several *Erythrina* species including: *E. corallodendron* (Gilboa-Garber and Mizrahi, 1981, Can. J. Biochem. 59, 315-320), *E. cristagalli* (Iglesias et al., 1982, Eur. J. Biochem. 123, 247-252), *E. indica* (Horejsi et al., 1980, Biochim. Biophys. Acta 623, 439-448), *E. arborescens*, *E suberosa*, *E. lithosperma* (Bhattacharyya et al., 1981, Archiv. Biochem. Biophys. 211, 459-470) *E. caffra*, *E. flabelliformis*, *E. latissima*, *E. lysistemon*, *E. humeana*, *E. perrieri*, *E. stricta*, and *E. zeyheri* (Lis et al., 1985, Phytochem. 24, 2803-2809).

These lectins have been analysed for their selectivity for saccharide binding (see e.g. Kaladas et al., 1982, Archiv. Biochem. Biophys. 217, 624-637). They have been found to

bind preferentially to oligosaccharides with a terminal β-D-galactosyl residue.

A second example of a plant-derived, galactose-binding lectin with the desired binding specificity can be obtained from *Glycine max* (soy) beans. This lectin (soya bean agglutinin, SBA) is a tetrameric protein with a total molecular weight of approximately 110 kDa. It binds to oligosaccharides containing galactose or N-acetylgalactosamine residues.

An example of a galactose-binding lectin from bacteria is PA-I, obtained from *Pseudomonas aeruginosa*. PA-I is a D-galactosephilic lectin with a molecular weight of about 13 kDa and it binds to galactose-containing oligosaccharides (Gilboa-Garber and Mizrahi, 1981, Can. J. Biochem. 59, 315-320).

These and other lectins of the sub-class of galactose-binding lectins can be used as targeting moieties (TM) for conjugates of the type described in WO96/33273. The requirements for TMs in these agents are that they show specificity for the primary sensory afferents over other spinal nerves and that they lead to the internalisation of the agents into an appropriate intracellular compartment. The lectins of this invention fulfil these criteria. Surprisingly, in comparison to other lectins of WO96/33273, they can fulfil these criteria more efficiently and can provide agents with enhanced selectivity for nociceptive afferent neurosecretion.

Thus, in one embodiment of the invention a galactose-binding lectin is conjugated, using linkages that may include one or more spacer regions, to a derivative of the clostridial neurotoxins.

In another embodiment of the invention the agent is expressed in a recombinant form as a fusion protein. The fusion protein may be derived from nucleic acid encoding an appropriate fragment of a galactose-binding lectin, in addition to any desired spacer domains, with nucleic acid encoding all or part of a polypeptide of one serotype of neurotoxin. Such a nucleic acid may be a chimera derived from the nucleic acid encoding polypeptides from more than one serotype.

In another embodiment of the invention the required LH_N, which may be a hybrid of an L and H_N from different clostridial toxin serotypes, is expressed as a recombinant fusion protein with the galactose-binding lectin, and may also include one or more spacer regions.

In a further embodiment of the invention the required TM, L or LH_N and translocation domain components may be separately expressed in a recombinant form and subsequently linked, covalently or non-covalently, to provide the desired agent.

In a further embodiment of the invention the required translocation domain may be of a non-clostridial origin, comprising instead a peptide or other entity capable of similar or enhanced function. Examples would include, but not be restricted to, the translocation domain of diphtheria toxin (O'Keefe *et al.*, Proc. Natl. Acad. Sci. USA (1992) **89**, 6202-6206 ; Silverman *et al.*, J. Biol. Chem. (1993) **269**, 22524-22532), the translocation domain of *Pseudomonas* exotoxin type A (Prior *et al.* Biochemistry (1992) **31**, 3555-3559), the translocation domains of anthrax toxin (Blanke *et al.* Proc. Natl. Acad. Sci. USA (1996) **93**, 8437-8442) and a variety of fusogenic or hydrophobic peptides of translocating function (Plank *et al.* J. Biol. Chem. (1994) **269**, 12918-12924).

Exploitation in Industry

The agent described in this invention can be used *in vivo*, either directly or as a pharmaceutically acceptable salt, for treatment of pain.

For example, an agent according to the invention can be administered by spinal injection (epidural or intrathecal) at the level of the spinal segment involved in the innervation of an affected organ for the treatment of pain. This is, for example, applicable in the treatment of deep tissue pain, such as chronic malignant pain.

The present invention will now be described by reference to the following examples together with the Figures that show the following:

Figure 1. SDS-PAGE analysis of fractions from ExL-LH_N/A purification scheme

Figure 2 Cleavage of SNAP-25 by ExL-LH_N/A

Figure 3. SDS-PAGE analysis of fractions from EcL-LH_N/A purification scheme

Figure 4 SDS-PAGE analysis of fractions from SBA-LH_N/A purification scheme

Figure 5 Native gel analysis of ExL- and SBA-LH_N/A

Figure 6 Activity of ExL-LH_N/A on release of neurotransmitter from eDRG and eSC neurons

Figure 7 Activity of SBA-LH_N/A on release of neurotransmitter from eDRG and eSC neurons

Figure 8 Activity of WGA-LH_N/A on release of neurotransmitter from eDRG and eSC neurons

Figure 9 Activity of ExL-LH_N/A in an *in vivo* electrophysiology model of analgesia

Figure 10 Activity of ExL-LH_N/A in an *in vivo* behavioural model of analgesia

Example 1. The Production of a conjugate between a lectin from *Erythrina cristagalli* and LH_N/A.

Materials

Lectin from *E. cristagalli* (ExL) was obtained from Sigma Ltd.

LH_N/A was prepared essentially by the method of Shone C.C., Hambleton, P., and Melling, J. 1987, *Eur. J. Biochem.* **167**, 175-180.

SPDP was from Pierce Chemical Co.

PD-10 desalting columns were from Pharmacia.

Dimethylsulphoxide (DMSO) was kept anhydrous by storage over a molecular sieve.

Denaturing sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using gels and reagents from Novex

Immobilised lactose-agarose was obtained from Sigma Ltd.

Additional reagents were obtained from Sigma Ltd.

Methods

The lyophilised lectin was rehydrated in phosphate buffered saline (PBS) to a final concentration of 10 mg/ml. Aliquots of this solution were stored at -20°C until use.

The ExL was reacted with an equal concentration of SPDP by the addition of a 10 mM stock solution of SPDP in DMSO with mixing. After one hour at room temperature the reaction was terminated by desalting into PBS over a PD-10 column.

The thiopyridone leaving group was removed from the product by reduction with dithiothreitol (DTT, 5 mM, 30 min). The product of this reaction was analysed spectrophotometrically at 280 nm and 343 nm to determine the degree of derivatisation achieved. The degree of derivatisation achieved was 0.8±0.06 mol/mol. The thiopyridone and DTT were removed by once again desalting into PBS over a PD-10 column.

The LH_N/A was desalted into PBSE (PBS containing 1 mM EDTA). The resulting solution (0.5-1.0 mg/ml) was reacted with a four- or five-fold molar excess of SPDP by addition of a 10 mM stock solution of SPDP in DMSO. After 3 h at room temperature the reaction was terminated by desalting over a PD-10 column into PBS.

A portion of the derivatised LH_N/A was removed from the solution and reduced with DTT (5 mM, 30 min). This sample was analysed spectrophotometrically at 280 mm and 343 nm to determine the degree of derivatisation. The degree of derivatisation achieved was 2.26±0.10 mol/mol.

The bulk of the derivatised LH_N/A and the derivatised ExL were mixed in proportions such that the ExL was in greater than three-fold molar excess. The conjugation reaction was allowed to proceed for >16 h at 4°C.

The product mixture was centrifuged to clear any precipitate that had developed. The supernatant was concentrated by centrifugation through concentrators (with 10000-50000 molecular weight exclusion limit) prior to a two step purification strategy. As the first step, the concentrated material was applied to a Superose 12 column on an FPLC chromatography system (Pharmacia). The column

was eluted with PBS and the elution profile followed at 280 nm.

Fractions were analysed by SDS-PAGE on 4-20% polyacrylamide gradient gels, followed by staining with Coomassie Blue. The major band of conjugate has an apparent molecular mass of between 130-160 kDa; this is separated from the bulk of the remaining unconjugated LH_N/A and more completely from the unconjugated ExL. Fractions containing conjugate were pooled prior to the second chromatography step; immobilised lactose-agarose. Selected post-Superose-12 fractions were applied to PBS-washed lactose-agarose and incubated for 2 hours at 4°C to facilitate binding. Lectin-containing proteins (i.e. ExL-LH_N/A conjugate) remained bound to the agarose during subsequent washing with PBS to remove contaminants (predominantly unconjugated LH_N/A). ExL-LH_N/A conjugate was eluted from the column by the addition of 0.3M lactose (in PBS) and the elution profile followed at 280 nm. The fractions containing conjugate were pooled, dialysed against PBS, and stored at 4°C until use.

In figure 1 is illustrated the SDS-PAGE profile during different stages in the conjugate purification scheme. Lanes 2 and 3 indicate ExL lectin and LH_N/A respectively prior to conjugation. Lanes 4, 5 & 6 represent conjugation mixture, post-Superose-12 and post-lactose affinity chromatography samples respectively. Lane 6 is therefore indicative of the profile of the final conjugate material. Molecular weight markers are represented in lanes 1 & 7 with sizes indicated on the figure.

On the SDS-PAGE gel there are bands due to lectin alone in fractions containing the conjugate, this material is probably due to the non-covalent homo-dimeric nature of the ExL; where only one monomer of ExL is covalently attached to the LH_N/A the other is dissociated from the complex by the SDS in the electrophoretic procedure giving rise to these bands. The absence of free lectin monomers

was confirmed by native PAGE analysis and is illustrated in Figure 5. ExL-LH_N/A (lane 5) was analysed by non-denaturing PAGE. Samples were separated using 4-20% polyacrylamide gel for 6.75 hours, 125V, 4°C. The electrophoresis profile was compared to those of LH_N/A (lane 3) and ExL lectin only (lane 4). A range of marker proteins were analysed alongside; apoferritin (lane 6), β-amylase (lane 8), alcohol dehydrogenase (lane 7) and albumin (lane 9). Approximate molecular sizes are indicated.

Example 2. The production of a conjugate between a lectin from *Erythrina corallodendron* and LH_N/A.

The procedure for production of a conjugate between a lectin from *Erythrina corallodendron* and LH_N/A is essentially as described in Example 1 but with the following differences:

Materials

Lectin from *E. corallodendron* (EcL) was obtained from Sigma Ltd.

Figure 3 illustrates the purification scheme for the EcL-LH_N/A conjugate. Samples were applied to 4-20% polyacrylamide gradient gels and subjected to electrophoresis prior to staining with Coomassie blue. Lane 1 = molecular weight markers. Lane 2 represents the post-lactose affinity purified sample of EcL-LH_N/A. Lane 3 is a sample of pre-lactose affinity purified (size-exclusion chromatography only) EcL-LH_N/A. Lane 4 is a sample of pre-lactose affinity purified ExL-LH_N/A.

Example 3. The Production of a conjugate between a lectin from *Glycine max* and LH_N/A

The procedure for production of a conjugate between a lectin from *Glycine max*_and LH_N/A is essentially as described in Example 1 but with the following differences:

Materials

Lectin from *G. max* (SBA) was obtained from Sigma Ltd.

Method

For the affinity chromatography step an immobilised N-acetylgalactosamine (GalNAc) column was used and specific SBA-LH_N/A was eluted by the addition of 0.3M lactose. Figure 4 illustrates SDS-PAGE profile changes during the purification scheme for SBA-LH_N/A. SBA-LH_N/A was purified from crude conjugate mixture by Superose-12 size-exclusion chromatography and immobilised N-acetylgalactosamine affinity chromatography. Samples were subjected to SDS-PAGE on 4-20% polyacrylamide gels. Lanes 6-8 were run in the presence of 0.1M DTT. Lanes 1 (&7) and 2 (&8) indicate SBA and SPDP-derivatised LH_N/A respectively, prior to conjugation. Lanes 3, 4 & 5 (&6) represent conjugation mixture, post-Superose-12 and post-affinity chromatography samples respectively. Lane 5 is therefore indicative of the profile of the final conjugate material. Molecular weight markers are represented in lanes Mr with sizes indicated on the figure.

The absence of free lectin monomers was confirmed by native non-denaturing PAGE analysis as illustrated in Figure 5. Samples were separated using 4-20% polyacrylamide gel for 6.75 hours, 125V, 4°C. The electrophoresis profile of SBA-LH_N/A (lane 1) was compared to those of SBA lectin only (lane 2) and LH_N/A (lane 3). A range of marker proteins were analysed alongside; apoferritin (lane 6), β-amylase (lane 8), alcohol dehydrogenase (lane 7) and albumin (lane 9). Approximate molecular sizes are indicated.

Example 4. Activity of ExL-LH_N/A in primary neuronal cultures

The dorsal root ganglia contain the cell bodies of primary nociceptive afferent neurons. It is well established that in primary *in vitro* cultures of this tissue the neurons retain many of the characteristics of the nociceptive afferent. These characteristics include the ability to release neuropeptides such as substance P in response to chemical stimuli known to cause pain *in vivo* (e.g. capsaicin). Neurons anatomically adjacent to those of the DRG include those of the spinal cord. Cultures of SC neurons prepared from embryonic rats can be established *in vitro* and the release of neurotransmitter (^{3}H-glycine) under potassium stimulation can be assessed. As such, the eSC neurons represent a model cell for testing the selectivity of the agents described.

The selectivity of the ExL-LH_N/A agent for eDRG over eSC neurons is clearly illustrated in Figure 6. The dose curves document the effectiveness of ExL-LH_N/A in an *in vitro* cell culture model by comparing inhibition of neurotransmission in eDRG with eSC neurons.

Materials

Substance P enzyme linked immunosorbent assay kits were from Cayman Chemical Company.

Western blot reagents were obtained from Novex

Monoclonal antibody SMI-81 was from Sternberger Monoclonals Inc.

Methods

Primary cultures of dorsal root ganglion and embryonic spinal cord neurons were established following dissociation of the ganglia dissected from rat embryos (embryological age 12-15 days). For the preparation of

eDRG neurons, the cells were plated into 12 well plates at an initial density of 3 x 10^5 cells/well in a medium containing NGF (100 ng/ml). After one day in culture, fresh medium containing cytosine arabinoside (10 X10^{-6} M) was added to kill non-neuronal cells. After 2-4 days the cytosine arabinoside was removed. After several more days in culture the medium was replaced with fresh medium containing conjugate or LH_N.

For the preparation of eSC neurons, Cells were plated onto poly-D-lysine coated 12 well plates (Costar) at a density of 2x10^6 cells per well (1 ml/well). 'Plating' medium was MEM with Earles Salts (Sigma), containing 5% foetal bovine serum (FBS), 5% heat inactivated horse serum (HS), 0.6% dextrose, 1.5g/l $NaHCO_3$ and 2 mM L-glutamine. Cultures are incubated at 37°C with 10% CO_2. The medium was changed to 'feeding' medium (plating medium minus the FBS with N1 (Sigma) 1/50 supplement) after one day. When glial cells became almost confluent anti-mitotic agents (15 micrognammes /ml 5-fluoro-2'-deoxyuridine (FdU) and 35 micrognammes /ml uridine (U)) were added for a further 2-3 days. Cells were cultured for at least 3 weeks prior to use.

The cells were incubated with these agents for varying times and then tested for their ability to release the neurotransmitters glutamate and substance P (eDRG) or glycine (eSC). After the release assays were performed the cells were lysed and the hydrophobic proteins were extracted by phase partitioning with Triton-X-114 following the method outlined in Boyd, Duggan, Shone and Foster (J. Biol. Chem. **270**, 18216-18218, *1995*).

Substance P release assay

The release of endogenous substance P was effected by collecting cell supernatants after treating the cells for 5 min with either a physiological balanced salt solution

or a balanced salt solution in which the potassium ion concentration had been raised to 100 mM with consequent reduction in the sodium ion concentration to maintain isotonicity. Total substance P was measured after extraction in 2 M acetic acid, 0.1% trifluoroacetic acid and subsequent dehydration. Substance P immunoreactivity was measured using an enzyme immunoassay kit (Cayman Chemical Company).

[^{3}H]Glutamate release assay

The release of glutamate was measured after loading the cells with [^{3}H]glutamine as a radiotracer. The [^{3}H]glutamine is converted to [^{3}H]glutamate in the cell, and it is this [^{3}H]glutamate that is taken up by synaptic vesicles and released upon depolarisation of the neuron. The cells are loaded with the [^{3}H]glutamine (5 X10^{-6} Ci/ml in HEPES-buffered MEM) for 2 h, then washed twice with HEPES-buffered MEM and thrice with balanced salt solution (BSS). Basal release was assessed with a 3 min incubation with BSS. Stimulated release was determined by a 3 min incubation with BSS in which the potassium concentration had been elevated to 80-100 mM with a consequent reduction in the sodium concentration to maintain isotonicity. All manipulations were performed at 37°C. The cells were lysed by the addition of Triton-X-100 (0.1%, v/v). For the basal and stimulated release superfusates the glutamate was separated from the glutamine by ion-exchange chromatography over Dowex-1 resin. The relevant fractions were analysed for ^{3}H content by liquid scintillation counting.

[^{3}H] Glycine release assay

The release of glycine was measured after loading the cells with [^{3}H]glycine as a radiotracer. The [^{3}H]glycine is taken up by synaptic vesicles and released upon depolarisation of the neuron. The cells are loaded with

the [^{3}H]glycine (2 X10^{-6} Ci/ml in HEPES-buffered MEM) for 2 h, then washed once with HEPES-buffered MEM and thrice with balanced salt solution (BSS). Basal release was assessed with a 5 min incubation with BSS. Stimulated release was determined by a 5 min incubation with BSS in which the potassium concentration had been elevated to 56 mM with a consequent reduction in the sodium concentration to maintain isotonicity. All manipulations were performed at 37°C. The cells were lysed by the addition of 2 M acetic acid, 0.1% trifluoroacetic acid. Fractions were analysed for their ^{3}H content by liquid scintillation counting and inhibition of release determined.

Figure 6 illustrates the activity of ExL-LH$_N$/A on release of neurotransmitter from eDRG and eSC neurons. Both eDRG and eSC cultures were exposed to a range of ExL-LH$_N$/A concentrations (1 ml volumes) for three days. The percentage inhibition of eDRG substance P (n) and eSC [^{3}H]-glycine (?) release is in comparison to untreated controls. The data shown is representative of =3 determinations. IC$_{50}$ for eDRG was determined to be 3.66±0.92pg/ml. An inhibition of 50% was not obtained for eSC using the concentration range employed.

Western blotting

ExL-LH$_N$/A was applied to eDRG for 16 hours. After the determination of neurotransmitter release the cells were lysed by the addition of 2 M acetic acid, 0.1% trifluoroacetic acid and subsequently dehydrated. To extract the membrane proteins from these mixtures Triton-X-114 (10%, v/v) was added and incubated at 4°C for 60 min, the insoluble material was removed by centrifugation and the supernatants were then warmed to 37°C for 30 min. The resulting two phases were separated by centrifugation and the upper phase discarded. The proteins in the lower phase were precipitated with chloroform/methanol for analysis by Western blotting.

The extracted protein samples were applied to 4-20% polyacrylamide gradient gels and subjected to electrophoresis prior to transfer to nitrocellulose. Proteolysis of SNAP-25, a crucial component of the neurosecretory process and the substrate for the zinc-dependent endopeptidase activity of BoNT/A, was then detected by probing with an antibody (SMI-81) that recognises both the intact and cleaved forms of SNAP-25 (Figure 2). Proteins blotted onto nitrocellulose were probed with antibody SMI-81. Lanes 1-3, 4-6, 7-9 and 10-12 represent cells treated with medium, 40 microgrammes/ml ExL, 20 microgrammes/ml ExL and 40 microgrammes/ml LH_N/A respectively. Densitometric analysis of these data determined the %SNAP-25 cleavage to be 52.7% and 37.0% for 40 and 20 microgrammes/ml respectively.

Example 5. Activity of SBA-LH_N/A in primary neuronal cultures

Using methodology described in Example 4, the activity of SBA-LH_N/A in primary neuronal cultures was assessed. The selectivity of the SBA-LH_N/A conjugate for eDRG over eSC neurons is illustrated in Figure 7. Both eDRG and eSC cultures were exposed to a range of SBA-LH_N/A concentrations (1 ml volumes) for three days. The percentage inhibition of eDRG substance P (n) and eSC [^{3}H]-glycine (O) release is in comparison to untreated controls. The data is the mean of three determinations $\pm$ SE. The curves shown are representative of two experiments. IC_{50} values for eDRG neurons were determined to be 1.84 and 7.6 microgrammes/ml. It is observed that SBA-LH_N/A exhibits a clear selectivity of the inhibition of neurotransmitter release from eDRG relative to eSC neurons. These data therefore confirm observations described for ExL-LH_N/A above and highlight the properties of galactose-specific lectins.

Example 7. Activity of WGA-LH_N/A in primary neuronal cultures

Using methodology described in Example 4, the activity of WGA-LH_N/A in primary neuronal cultures was assessed. WGA represents an example of a non-galactosyl targeted lectin and therefore serves as an indicator of the properties of conjugate that do not recognise galactosyl moieties. The lack of selectivity of the WGA-LH_N/A conjugate for eDRG over eSC neurons is illustrated in Figure 8. eDRG and eSC neurons were exposed to a range of concentrations of WGA-LH_N/A for 3 days prior to assay of stimulated release of neurotransmitter (substance P and glycine respectively). Each conjugate concentration was assessed in triplicate and results are expressed as percentage inhibition compared to untreated controls. Panels A and B represent dose response curves from one experiment representative of ≥3 for eDRG and eSC neurons respectively. Each point shown is the mean of three determinations ± SE of the mean. IC_{50} data for the effects of WGA-LH_N/A was calculated to be 0.34±0.06 microgrammes /ml (eDRG) and 0.0010.03 microgrammes /ml (eSC), indicating the lack of C-fibre selectivity.

Example 8. Activity of ExL-LH_N/A in an electrophysiological model of pain

A dose of 45 microgrammes of ExL-LH_N/A in a 10 microlitres volume of vehicle was given by intrathecal injection to rats between lumbar sections L4-L5, 24 hours prior to electrophysiological analysis of neuronal activity. Animals were allowed to recover and movement was not restricted prior to sacrifice and analysis. The results from a group of 3 animals with 10 neurons recorded per animal, show that there was a 73% reduction in the C-fibre responses of the neurones (Figure 9A) although the stimulus threshold is only slightly elevated (Figure 9B). Inhibition of C-fibre responses would lead to a decrease

in the transmission of pain signals and these data are indicative of the analgesic effect of conjugate ExL-LH_N/A. There was also a significant decrease in the A_δ response (Figure 9C). These fibres are also implicated in the transmission of noxious stimuli and this result emphasises the analgesic effect of ExL-LH_N/A. A_β neurons, a cell type that is not involved in transmission of noxious stimuli, were essentially unaltered in their responses to this stimulus (Figure 9D). The lack of affect on the A_β-fibre neurons is indicative of the selectivity of ExL-LH_N/A for the neurons central to the transmission of pain signals.

Example 9. Activity of ExL-LH_N/A in behavioural models of pain

In an accepted *in vivo* model of pain, the mouse hotplate test, ExL-LH_N/A has been demonstrated to exhibit analgesic properties. Figure 10 illustrates the data obtained for ExL-LH_N/A where it is compared to a supramaximal dose of morphine. ExL-LH_N/A was applied intrathecally (30 microgrammes in a 5 microlitre vehicle volume) to each of a group of 10 mice and analgesic response in the hot plate test determined. Data is presented as hot plate latency (seconds) plotted against assay time (P = pre-treatment, 0-5 = hours post application). Onset of ExL-LH_N/A action had apparently reached a plateau at 1 hour that remained constant for at least 5 hours. The level of analgesia is similar to a supramaximal dose (50 microgrammes, 20X mouse EC_{50}) of morphine in this test, but is of much longer duration. This level of morphine achieves a maximal effect at 1 hour and then returns to control levels over a period of 5 hours. These data represent a clear indication of the analgesic potential of agents such as ExL-LH_N/A.

Materials

Adult outbred mice (MF1) of either sex, weight range 20 to 30g.

Methods

Test material is injected into the intrathecal space of anaesthetised mice using a 30 gauge disposable needle attached to a 50 microlitre Hamilton syringe. The site of injection was normally chosen to be between lumbar vertebrae 5 and 6. The needle is inserted into the tissue to one side of the vertebrae so that it slips into the groove between the spinous and transverse processes. The needle is then moved carefully forward to the intervertebral space. 5 microlitres of test material is then injected into the intrathecal space and the needle withdrawn. The skin incision is then closed with a single wound clip and the animal placed in a box to allow recovery.

Claims

1. An agent for the treatment of pain that comprises a galactose-binding lectin linked to a derivative of a clostridial neurotoxin, in which the derivative of the clostridial neurotoxin comprises the L-chain, or a fragment thereof, which includes the active proteolytic enzyme domain of the light (L) chain, linked to a molecule or domain with membrane translocating activity.

2. An agent according to Claim 1 in which the membrane translocation domain is derived from the heavy chain of a clostridial toxin.

3. An agent according to Claim 1 in which the membrane translocation domain is derived from a non-clostridial source.

4. An agent according to any preceding Claim in which the lectin binds to oligosaccharides that contain terminal β-D-galactosyl residues

5. An agent according to any preceding Claim in which the lectin binds to oligosaccharides that contain terminal α-D-galactosyl residues

6. An agent according to any preceding Claim in which the lectin binds to oligosaccharides that contain N-acetylgalactosamine

7. An agent according to any previous Claim in which the lectin is derived from a species of plant.

8. An agent according to the previous Claim in which the lectin is derived from a species of the genus *Erythrina*.

9. An agent according to Claim 8 in which the lectin is derived from *E. cristagalli*.

10. An agent according to Claim 8 in which the lectin is derived from *E. corallodendron*.

11. An agent according to Claims 7 in which the lectin is obtained from *Glycine max*.

12. An agent according to Claims 7 in which the lectin is obtained from *Arachis hypogaea*.

13. An agent according to Claims 7 in which the lectin is obtained from *Bandeirea simplicifolia*.

14. An agent according to Claim 1-6 in which the lectin is of mammalian origin.

15. An agent according to Claim 1-6 in which the lectin is obtained from bacteria.

16. An agent according to Claim 15 in which the lectin is obtained from *Pseudomonas aeruginosa*.

17. An agent according to any preceding Claim in which the lectin has been produced using recombinant technology.

18. An agent according to any preceding Claim in which the lectin has been enzymatically modified.

19. An agent according to any preceding Claim in which the lectin has been chemically modified.

20. An agent according to any preceding Claim which comprises the lectin coupled to a clostridial neurotoxin in which the H_C domain of the H-chain is removed or modified.

21. An agent according to any preceding Claim in which the H-chain is modified by chemical derivatisation to reduce or remove its native binding affinity for motor neurons.

22. An agent according to any of Claims 1-20 in which the H-chain is modified by mutation to reduce or remove its native binding affinity for motor neurons.

23. An agent according to any of Claims 1-20 in which the H-chain is modified by proteolysis.

24. An agent according to Claim 20 in which the H_C domain is completely removed leaving only the H_N-fragment of a clostridial neurotoxin.

25. An agent according to any preceding Claim in which the clostridial neurotoxin component is obtained from botulinum neurotoxin.

26. An agent according to any preceding Claim in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type A.

27. An agent according to Claims 1-25 in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type B.

28. An agent according to any of Claims 1-25 which is formed by the coupling of a galactose-binding lectin to the LH_N fragment of botulinum neurotoxin type A.

29. An agent according to Claim 28 which is formed by the coupling of the galactose-binding lectin from *Erythrina cristagalli* to the LH_N fragment of botulinum neurotoxin type A.

30. An agent according to Claim 28 which is formed by the coupling of the galactose-binding lectin from *Erythrina corallodendron* to the LH_N fragment of botulinum neurotoxin type A.

31. An agent according to Claim 28 which is formed by the coupling of the galactose-binding lectin from *Glycine max* to the LH_N fragment of botulinum neurotoxin type A.

32. An agent according to any preceding Claim in which the H-chain is obtained from a different clostridial neurotoxin than that from which the L-chain is obtained.

33. An agent according to Claim 32 in which the H-chain is obtained from botulinum neurotoxin type A and the L-chain from botulinum neurotoxin type B.

34. An agent according to Claim 32 in which the H-chain is obtained from botulinum neurotoxin type A and the L-chain from tetanus neurotoxin.

35. An agent according to Claims 33 and 34 in which the H-chain component is the H_N fragment of botulinum neurotoxin type A.

36. An agent according to any preceding Claim in which the L-chain or L-chain fragment is linked to the H-chain by a direct covalent linkage.

37. An agent according to any of Claims 1-35 in which the L-chain or L-chain fragment is linked to the H-chain by a covalent linkage which includes one or more spacer regions.

38. An agent according to any preceding Claim in which the clostridial neurotoxin derivative incorporates polypeptides produced by recombinant technology.

39. An agent according to any preceding Claim in which the lectin is linked to the clostridial neurotoxin-derived component by a direct covalent linkage.

40. An agent according to any of Claims 1-38 in which the lectin is linked to the clostridial neurotoxin-derived component by a covalent linkage which includes one or more spacer regions.

41. An agent according to any preceding Claim in which the lectin and clostridial neurotoxin components are produced as a recombinant fusion protein.

42. An agent according to any preceding Claim in which the lectin protein has been modified from its native polypeptide sequence whilst retaining an ability for the protein to bind to oligosaccharide structures, in which the terminal residue is derived from galactose or N-acetylgalactosamine.

43. An agent according to Claim 42 in which the protein modification results from modification of the nucleic acid coding for the lectin protein from its native sequence.

44. An agent according to any preceding Claim which prevents the release of a neurotransmitter or neuromodulator from a primary sensory afferent.

45. An agent according to any preceding Claim which inhibits the release of a neurotransmitter or neuromodulator from a primary nociceptive afferent.

46. A method for obtaining an agent according to any preceding Claim which comprises the covalent attachment of a galactose-binding lectin to a derivative of a clostridial neurotoxin, in which the derivative of the clostridial neurotoxin comprises the L-chain or an L-chain fragment which includes the active proteolytic enzyme domain of the light (L) chain, linked to a molecule or domain with membrane translocating activity.

47. A method for obtaining an agent according to any of Claims 1-58 which comprises the covalent attachment of a galactose-binding lectin to a derivative of a clostridial neurotoxin with the inclusion of one or more spacer regions, in which the derivative of the clostridial neurotoxin comprises the L-chain or an L-chain fragment which includes the active proteolytic enzyme domain of the light (L) chain, linked to a molecule or domain with membrane translocating activity.

48. An method according to Claim 47 in which the membrane translocation domain is derived from the heavy chain of a clostridial toxin.

49. An method according to Claim 47 in which the membrane translocation domain is derived from a non-clostridial source.

50. A method for obtaining an agent according to any of Claims 1-45 which comprises constructing a genetic construct which codes for the agent, incorporating said construct into a host organism and expressing the construct to produce the agent.

51. A method of controlling the release of a neurotransmitter or neuromodulator from a primary sensory afferent by applying the agent of any one of Claims 1-45.

52. A method of controlling the release of a neurotransmitter or neuromodulator from a primary nociceptive afferent by applying the agent of any one of Claims 1-45.

53. A method of controlling the transmission of sensory information from a primary sensory afferent to a projection neuron by applying the agent of any one of Claims 1-45.

54. A method of controlling the transmission of sensory information from a primary nociceptive afferent to a projection neuron by applying the agent of any one of Claims 1-45.

55. A method of controlling the sensation of pain by applying the agent of any one of Claims 1-45.

56. Use of the agent according to any one of Claims 1-45 or a pharmaceutically acceptable salt thereof as a medicament for the alleviation of pain.

57. Use of the agent according to any one of Claims 1-45 or a pharmaceutically acceptable salt thereof as a medicament for the prevention of pain.

58. Use of the agent according to any one of Claims 1-45 in the manufacture of a medicament for the alleviation of pain.

59. Use of the agent according to any one of Claims 1-45 in the manufacture of a medicament for the prevention of pain.

60. A method of alleviating pain which comprises administering an effective dose of the agent according to any one of Claims 1-45.

61. A method of preventing pain which comprises administering an effective dose of the agent according to any one of Claims 1-45.

Figure 1

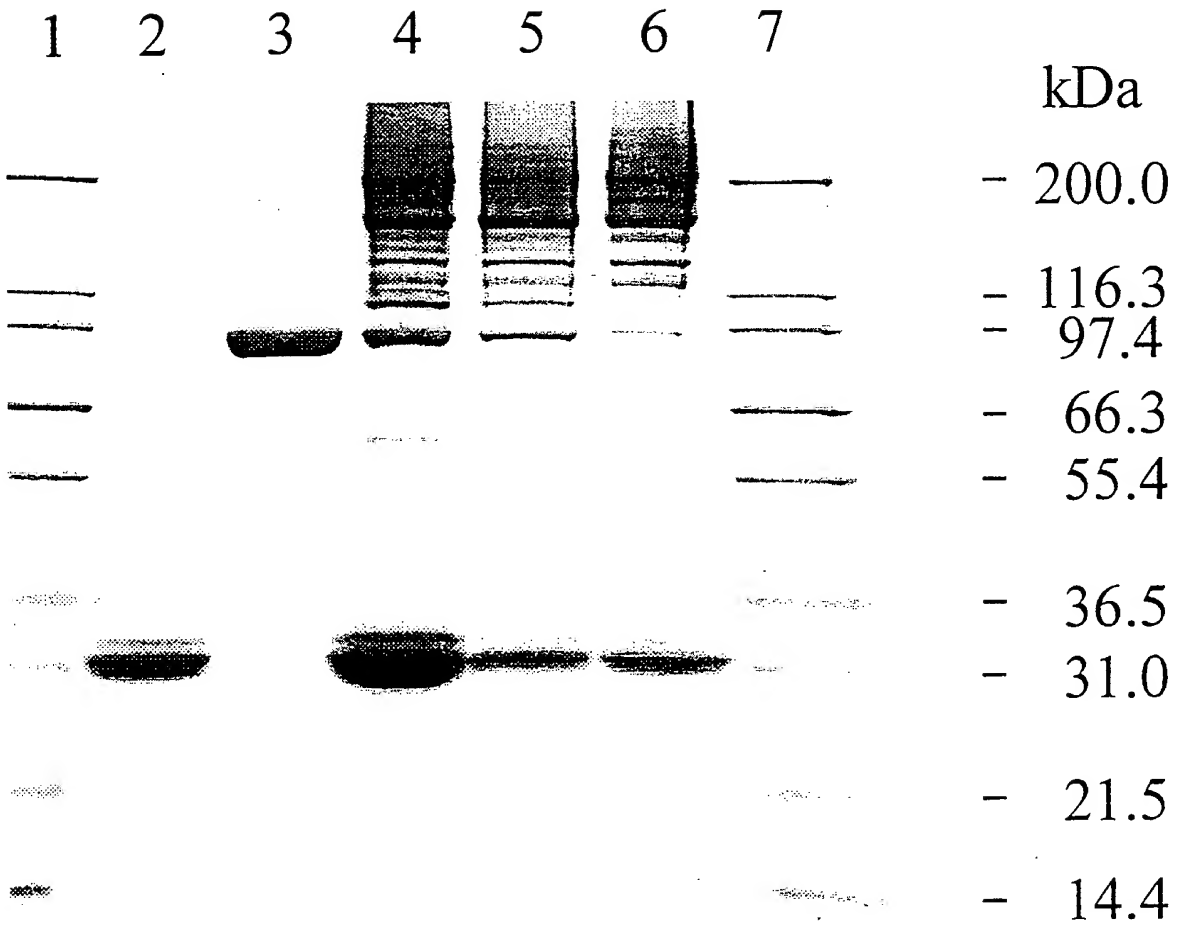


Figure 2

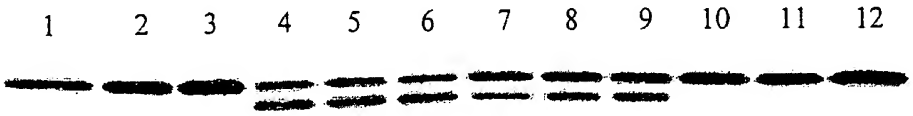


Figure 3

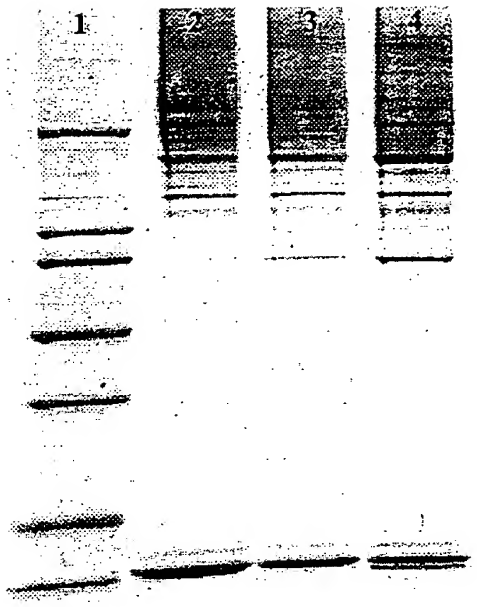


Figure 4

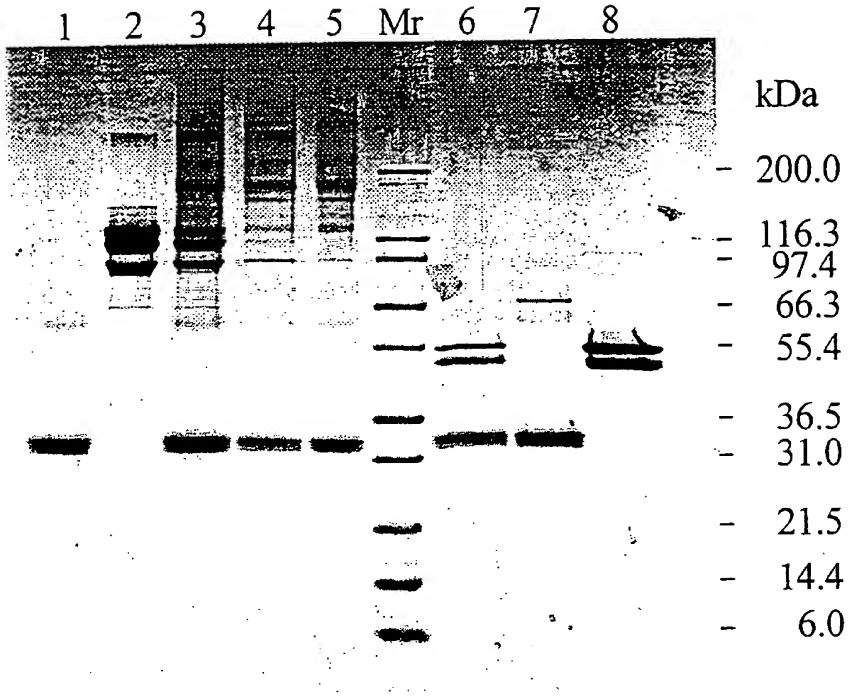


Figure 5

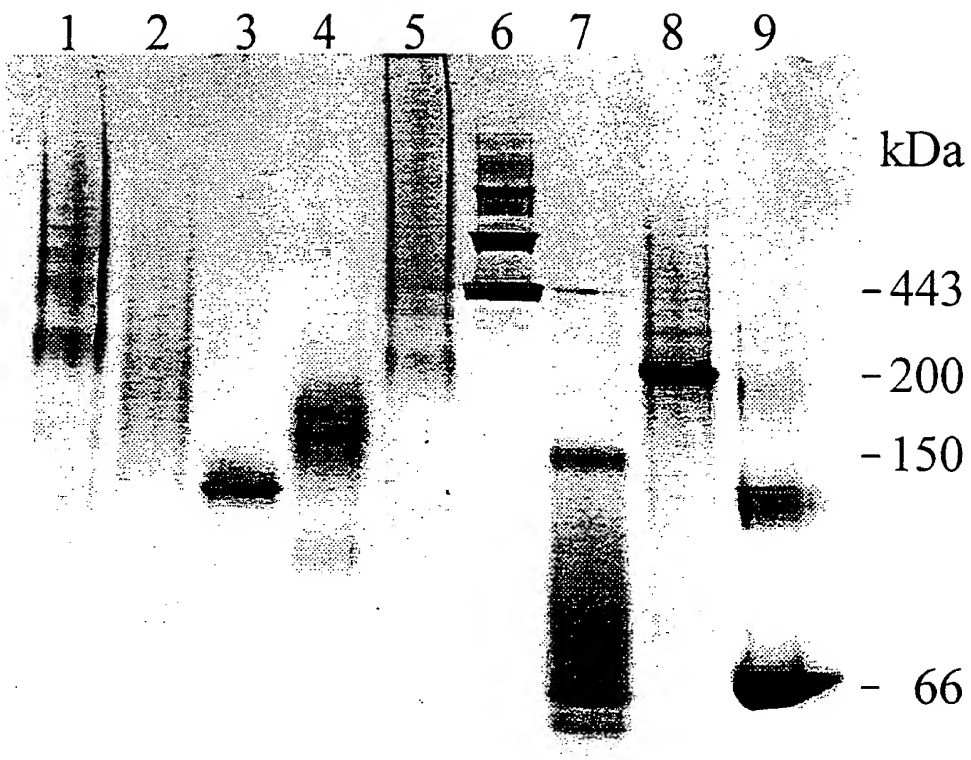
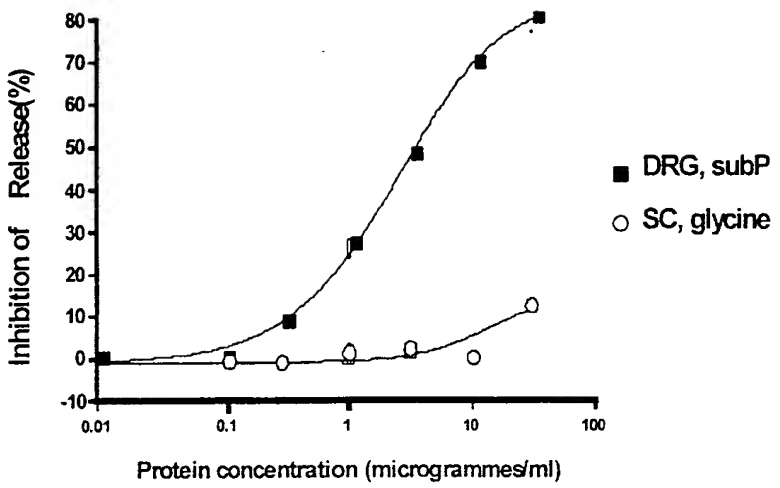


Figure 6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60
50
40
30
20
10
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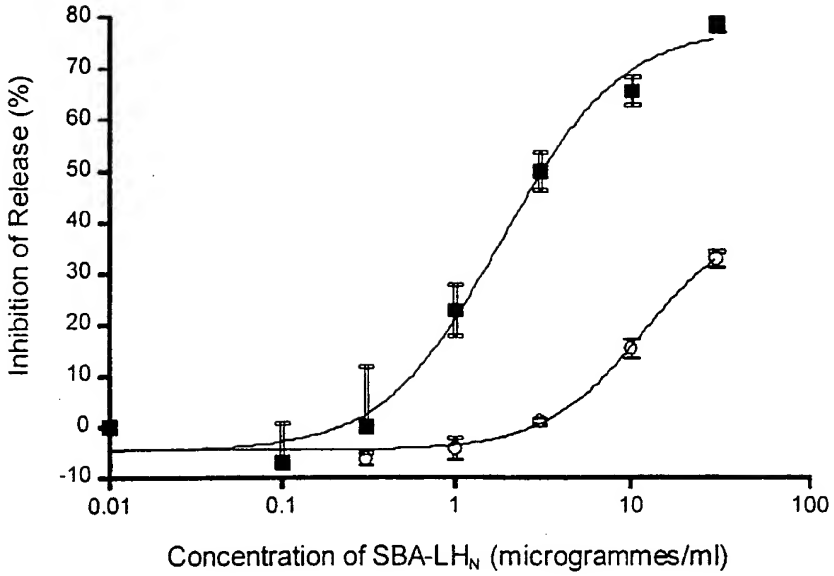
Protein concentration (microgrammes/ml)
DRG, subP
SC, glycine

Figure 7


Inhibition of Release (%)
80
70
60
50
40
30
20
10
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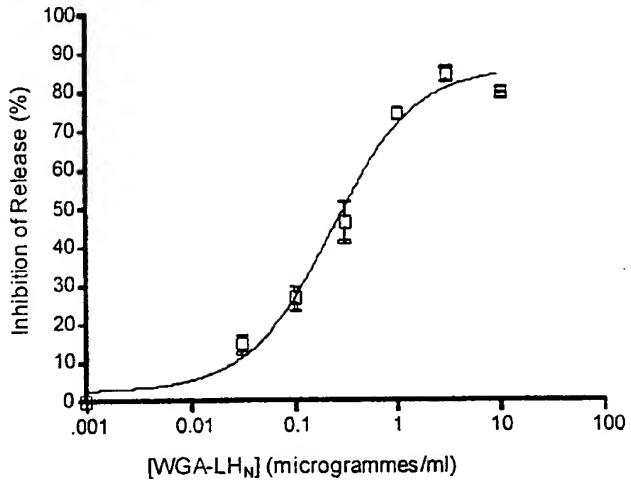
0.1
1
10
100
Concentration of SBA-LH$_N$ (microgrammes/ml)

Figure 8

Panel A: eDRG



Panel B: eSC neurons

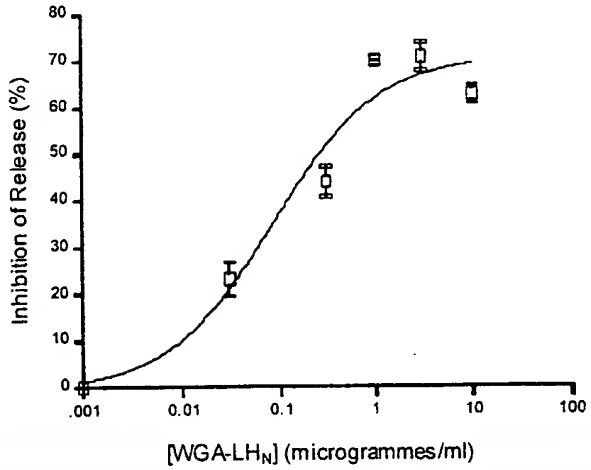
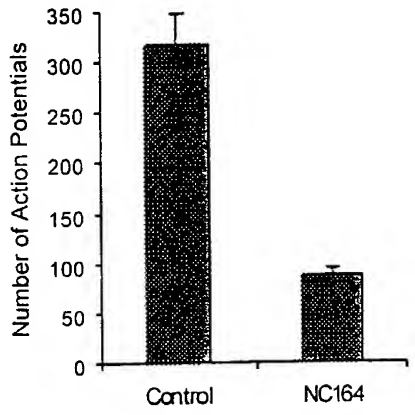


Figure 9

A.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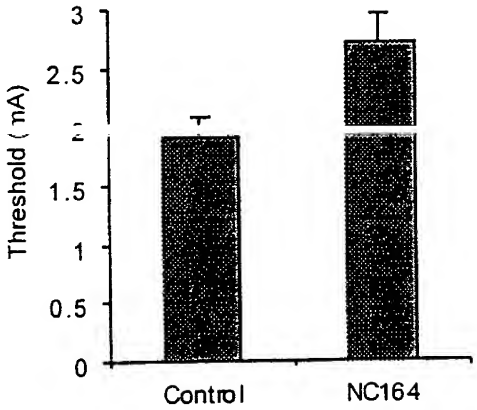
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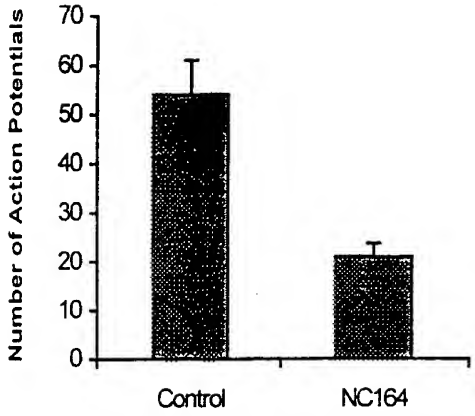
C.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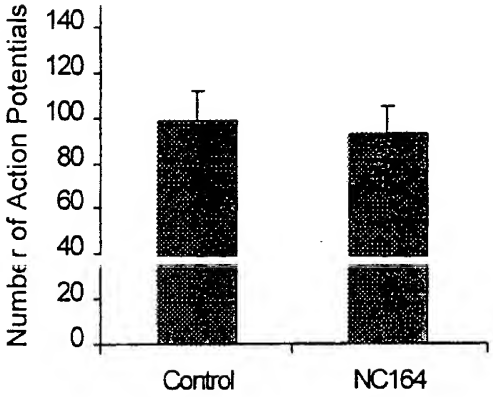
Control
NC164

D.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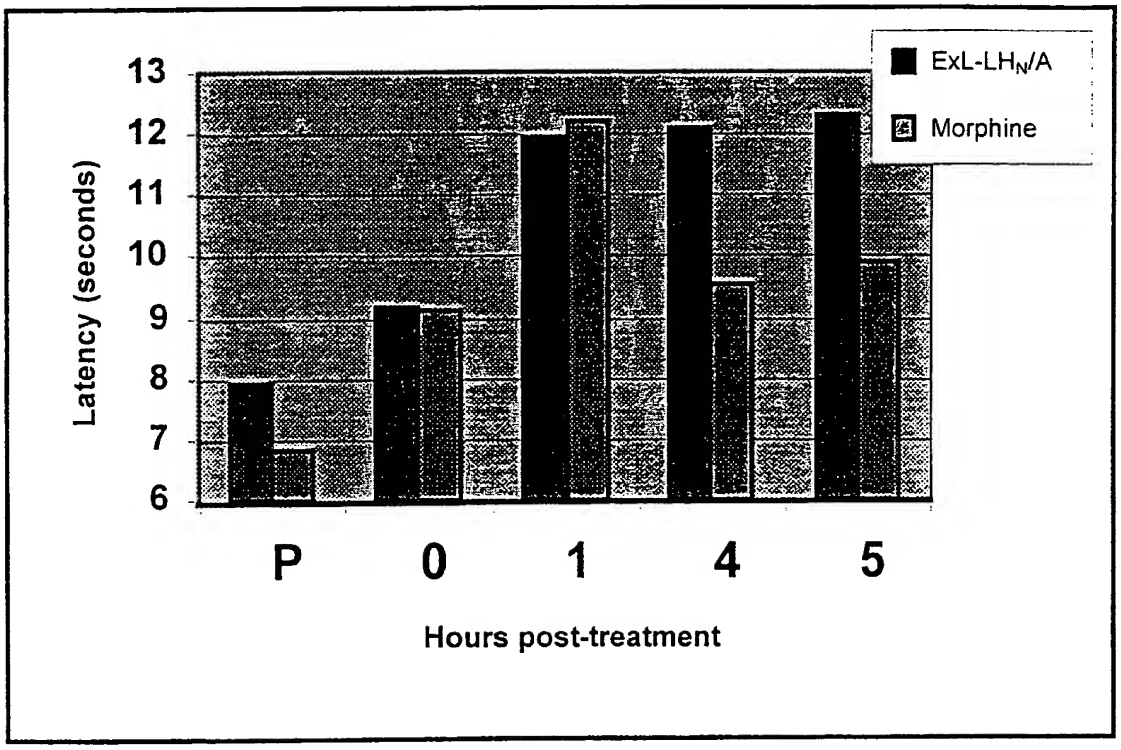
NC164

Figure 10


ExL-LH$_N$/A
Morphine
Latency (seconds)
13
12
11
10
9
8
7
6
P
0
1
4
5
Hours post-treatment

INTERNATIONAL SEARCH REPORT

Int. .tional Application No
PCT/GB 98/03001

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 33273 A (SPEYWOOD LAB LIMITED ;MICROBIOLOGICAL RES AUTHORITY (GB); FOSTER K) 24 October 1996 cited in the application	1-61
Y	see page 12, line 1 - page 13, line 8; claims see page 13, line 9 - line 13	1-61

P,X	WO 98 07864 A (FOSTER KEITH ALAN ;MICROBIOLOGICAL RES AUTHORITY (GB); QUINN CONRA) 26 February 1998 see claims	1

A	WO 94 28923 A (ALLERGAN INC) 22 December 1994 see claims	

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 January 1999	09/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Berte, M

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/03001

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 18790 A (UNIV MONTANA RES DEV INST ;PASCUAL DAVID (US); BOND CLIFFORD (US);) 29 May 1997 see claims 1,4,5,9,29,30 ---	1-61
A	WO 95 32738 A (ALLERGAN INC ;DOLLY JAMES OLIVER (GB); AOKI KEI ROGER (US); WHEELE) 7 December 1995 ---	
Y	STREIT, WOLFGANG J. ET AL: "Histochemical localization of galactose-containing glycoconjugates in sensory neurons and their processes in the central and peripheral nervous system of the rat" J. HISTOCHEM. CYTOCHEM. (1985), 33(10), 1042-52 CODEN: JHCYAS;ISSN: 0022-1554, 1985, XP002091075 see abstract -----	1-61

2

INTERNATIONAL SEARCH REPORT

...ernational application No.
PCT/GB 98/03001

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 51-55,60-61 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

In ational Application No
PCT/GB 98/03001

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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(54) Title: NOVEL AGENT FOR CONTROLLING CELL ACTIVITY

(57) Abstract

This invention describes a novel agent for the targeted control of a mammalian cell activity, in particular the agent is used to control the interaction of particular cell types with their external environment. The agent has applications as a pharmaceutical for the treatment of a variety of disorders. An agent according to the invention comprises three Domains B, T and E linked together in the following manner: Domain B--Domain T--Domain E where Domain B is the Binding Domain which binds the agent to a Binding Site on the cell which undergoes endocytosis to produce an endosome, Domain T is the Translocation Domain which translocates the agent (with or without the Binding Site) from within the endosome across the endosomal membrane into the cytosol of the cell, Domain E is the Effector Domain which inhibits the ability of the Recyclable Membrane Vesicles to transport the Integral Membrane Proteins to the surface of the cell.

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NOVEL AGENT FOR CONTROLLING CELL ACTIVITY

TECHNICAL FIELD

This invention describes a novel agent for the targeted control of mammalian cell activity, in particular the agent is used to control the interaction of particular cell types with their external environment. The agent has applications as a pharmaceutical for the treatment of a variety of disorders.

BACKGROUND

A fundamental property of living cells is their ability to respond to their external environment. The interface between a cell and its external environment is the plasma membrane. The plasma membrane consists of a phospholipid bilayer in which many kinds of protein molecules are embedded. These integral membrane proteins (IMPs) are responsible for many of the interactions of a cell with its external environment.

The interactions in which the IMPs are involved include: the transport of materials, including nutrients, into and out of the cell; the regulated permeability of the plasma membrane to ions; the recognition of, and response to, extracellular molecules; and the adhesion of one cell to another cell. A specialised function of the immune system, that is also mediated via IMPs, is the display of particular foreign peptide sequences by one group of immune cells to another group.

One of the ways in which a cell regulates its ability to respond to, and interact with, the external environment is by changing the quantity and types of IMPs present at the plasma membrane. One mechanism by which this is achieved is the reversible internalisation of IMPs via an endocytotic pathway into Recyclable Membrane Vesicles (RMVs). In these cases IMPs stored in the RMVs represent an internal store or pool of IMPs available for rapid export to the cell surface via a process of exocytotic fusion of the RMVs with the plasma membrane. Modulation of the equilibrium of this exocytotic / endocytotic

cycle allows rapid regulation of the density of IMPs present at the cell surface. In one example of the process for controlling cell activity, the uptake of glucose by insulin-responsive cells in skeletal muscle and adipose tissue is regulated. Insulin increases the amount of a particular isoform of glucose transporter, GLUT4, which is found in the plasma membrane of these cells. The higher concentration of GLUT4 molecules at the surface of the cell results in increased uptake of glucose. Therefore, by controlling the number of glucose transporters present in the plasma membrane the response to insulin can be modulated.

Another example of alterations in cell surface IMP expression in response to external signals is that of the receptor for the complement fragments C3b and C4b, the type 1 complement receptor CR1. Upon activation of human neutrophils the plasma membrane expression of CR1 is transiently increased 6- to 10- fold.

In another example a number of inflammatory and immune cells modify their expression of cell surface adhesion molecules upon activation. Hence, activation of neutrophils or monocytes leads to a modulation of the cell surface adhesion molecules Mac-1 and p150,95. These adhesion molecules are important in the targeting and movement of inflammatory cells to sites of inflammation.

In yet another example, a variety of hormones (insulin, insulin-like growth factor, interleukin 1 and platelet-derived growth factor) cause a rapid increase in the cell surface expression of the transferrin receptor in a variety of cell types. The transferrin-receptor binds diferric transferrin from the external environment of the cell, and is thereby involved in the uptake of iron by cells. This transferrin/transferrin-receptor system may also play a role in the transcellular movement of iron into the CNS across the blood brain barrier, a process known as transcytosis. Transcytosis is also involved in the transfer of maternal immunity to the developing foetus.

In yet another example the diuretic hormone aldosterone is known

to increase the cell surface expression of Na^+ channels in the apical membrane of urinary bladder epithelial cells. This mechanism is involved in salt retention and occurs, for example, in conditions of low sodium-containing diets.

In a further example of the modulation of cell membrane expression of IMPs, it is noted that the function of the immune system is based upon the recognition of foreign, or non-self, antigens. Part of this recognition and immune response is provided by cells of the immune system able to recognise and respond to foreign peptide sequences. These peptide sequences are presented to the immune cells by other cells of the immune system known as antigen presenting cells. Antigen presenting cells ingest foreign antigenic proteins, digest these to peptides and display the foreign peptides in a cleft formed at the cell membrane by IMPs of the major histocompatibility complex.

Thus IMPs are central to a cell's ability to interact with its external environment and, given the diverse and varied nature of these interactions, it is not surprising to discover that there are a vast array of different IMPs. The pivotal role of IMPs in a cell's function means that they are often involved in pathophysiologic states, and are the target for many therapeutic interventions.

Prior art approaches to the control of IMP activity have mainly focused on modulating the function of the IMP once expressed at the cell surface. Thus prior art therapeutic interventions tend to be specific for particular IMPs and for particular functions of particular cell types. Inhibitors of specific transport IMPs have been developed as therapeutic agents. For example, inhibitors of the 5HT transport protein of neurones are used as anti-depressants. Antagonists of particular receptor IMPs are very commonly used pharmaceutical agents. Examples include antihistamines, both those specific for the H1 and the H2 subtypes of histamine receptor, and antagonists of the ß-adrenoceptor. Inhibitors of IMP function are also widely used as pharmaceutical agents. Examples include inhibitors of

transmembrane ion movements such as the diuretics furosemide and amiloride, the latter of which is an inhibitor of the bladder epithelial cell apical Na^+ channel. Inhibitors of potassium channels are known to be under development as antiarrhythmic agents. Cell adhesion IMPs are also currently targets for the development of selective antagonists.

Another approach being pursued is to selectively modify the expression of particular IMPs at the genetic level by alteration of the level of transcription of the appropriate gene coding for that IMP and hence modulation of specific IMP protein synthesis.

In summary, IMPs are known to play a critical role in the response of a cell to its external environment. Previous approaches to the control of IMPs have generally involved the targeting of a specific IMP at the cell surface and modifying its functional capacity. The control of the density of IMPs within the plasma membrane is anticipated to have broad applications in the treatment of a variety of disorders. In view of the great diversity of IMPs and the particular nature of current therapeutic interactions it is the surprising discovery of the current invention that a single class of agents can modify the expression of IMPs in a wide variety of cell types. The same class of agent is also able to modify the expression of transport IMPs, receptor IMPs, adhesion IMPs, channel IMPs and antigen presenting IMPs. Previously, agents affecting IMPs have been classified by function, for example Ca^{++} antagonists, the members of each group being chemically and mechanistically very diverse. The class of agent referred to in the current invention, by contrast, is structurally homogeneous, with rationally introduced substitutes of particular domains having predictable effects on the function of the agent. A further aspect of the invention is that the agent can be selectively targeted to particular types of cell to allow selective modulation of IMP expression only in that cell type.

STATEMENT OF INVENTION

The current invention relates to an agent for controlling the

interaction of a cell with its external environment. Specifically, the invention provides an agent for controlling the transport of Integral Membrane Protein (IMP) molecules from the internal components of a cell to the cell membrane, so as to modify the cell's interaction with its external environment. More specifically the invention provides a novel agent which modifies the structure of Recyclable Membrane Vesicles (RMVs) such that their ability to transport IMPs to the surface of the cell is inhibited.

Definitions

The following terms have the following meanings;

Integral Membrane Protein (IMP) means any protein which is embedded in and spans across the lipid bilayer of a biological membrane

Recyclable Membrane Vesicle (RMV) means an intracellular vesicle, present in the cytosol of a cell, bounded by a lipid bilayer membrane . RMVs are formed from the plasma membrane and move into the cell interior by a process referred to as endocytosis. RMVs undergo a cyclical process of forming from and fusing with the cell plasma membrane. The process of moving to and fusing with the plasma membrane is referred to as exocytosis. The function of RMVs in the cell is in the reversible transport of IMPs to and from the cell surface; in this they are distinct from the secretory vesicles of neurosecretory cells.

Endosome means those intracellular vesicles which have formed from the plasma membrane by a process of endocytosis.

Heavy chain means the larger of the two polypeptide chains which form Clostridial neurotoxins; it has a molecular mass of approximately 100 kDa and is commonly referred to as HC. Light chain means the

smaller of the two polypeptide chains which form Clostridial neurotoxins; it has a molecular mass of approximately 50 kDa and is commonly referred to as LC. Naturally occurring Heavy and Light chains are covalently coupled via at least one disulphide bond.

H_2 fragment means a fragment derived from the amino terminal end of the Heavy chain of a Clostridial neurotoxin by proteolytic cleavage for example with trypsin or papain.

H_2L means a fragment of a Clostridial neurotoxin produced by proteolytic cleavage for example with trypsin or papain in which the Light chain is still coupled via disulphide bonds to the H_2 fragment.

In one aspect of the invention an agent is provided for the control of the level of the IMP responsible for the transport of a metabolite across the cell membrane, so controlling the availability of that metabolite within the cell.

In another aspect of the invention an agent is provided for the control of the level of the IMP responsible for the transport of a metabolite across the cell membrane into the cell and out of the cell, so controlling the transport of that metabolite through the cell.

In yet another aspect of the invention an agent is provided for the control of the level of the IMP responsible for the selective permeability of the plasma membrane of the cell to an ion, so modulating the concentration of that ion within the cell.

In yet another aspect of the invention an agent is provided for the control of the level of the IMP responsible for the recognition of a signalling molecule, so modulating the responsiveness of the cell to that signalling molecule.

In yet another aspect of the invention an agent is provided for

the control of the level of the IMP responsible for the transduction of signals across the cell membrane following binding to the membrane of a signalling molecule, so modulating the responsiveness of the cell to that signalling molecule.

In yet another aspect of the invention an agent is provided for the control of the level of the IMP responsible for the display on the cell surface of peptide fragments derived from ingested antigens. The result of this in an organism is to affect the immune response of that organism.

The invention also provides an agent which has target specificity for target cell types so that the scope of the effect of the agent is limited to said cell types.

As previously stated, prior art approaches to the control of IMP activity have mainly focused on modulating the function of the IMP once expressed at the cell surface. In direct contrast the present invention modulates the level of IMP which becomes expressed at the cell surface.

DETAILED DESCRIPTION OF THE INVENTION

It can be seen that the object of this invention, to provide an agent for controlling the level of IMPs at a cell surface, has many potential applications for modulating the response of a cell to its environment. This invention includes an agent which functions so as to affect the mechanisms by which IMPs are carried to the surface of a cell, as evidenced in the examples, e.g. example 1 and 2. Such an agent must accomplish three discrete functions, the first two of which are known in the art. Firstly it must bind to a cell surface structure (the Binding Site). It must then enter into the cytosol of the cell. The entry of molecules into the cell is known to occur by a process of endocytosis. However, as only certain cell surface Binding Sites are known to be involved in endocytosis, only these Binding Sites are suitable as targets. Once taken into the cell by endocytosis the agent must then leave the resulting endosome across the endosomal membrane to enter the cytosol. The ability to achieve specific cell binding and entry of agents into the

cytosol is well known in the literature (for example: Pastan, I; Willingham, MC; & Fitzgerald, DSP, 1986, Cell 47, 641 - 648, Olsnes, S; Sandvig, K; Petersen, OW; & Van Dews, B, 1989, Immunol. Today 10, 291 - 295, Strom, TB; Anderson, PL; Rubin-Kelley, VE; Williams, DP; Kiyokawa, T; & Murphy, JR; 1991, Ann NY Acad. Sci 636, 233 -250). The third function of the agent is the surprising finding of this invention, namely the ability to affect the RMV. The further surprising aspect of this agent is that by so affecting the RMV it limits its ability to transport the IMPs to the cell surface.

The agent of the invention therefore comprises the following functional Domains;

> Domain B, the Binding Domain, binds the agent to a Binding Site on the target cell capable of undergoing endocytosis to produce an endosome containing the agent
>
> Domain T, the Translocation Domain, translocates the agent or part of the agent from within the endosome across the endosomal membrane into the cytosol of the cell
>
> Domain E, the Effector Domain, inhibits transport of IMPs to the surface of the cell by RMVs.

Domain B can be made to have specificity for a target cell type. The ability to target an agent to a particular cell type is well known in the art. Thus, the functions of Domain B could be achieved by the use of one of many cell-binding molecules known in the art including, but not limited to, antibodies, monoclonal antibodies, antibody fragments (Fab, $F(ab)'_2$, Fv, single chain antibodies, etc.), hormones, cytokines, growth factors and lectins.

The functions of Domain T could be achieved by molecules capable of forming appropriate pores within the endosomal membrane. It is well documented that certain parts of toxin molecules are

capable of forming such pores including, amongst others, fragments of anthrax toxin, botulinum toxin, tetanus toxin, Diphtheria toxin and Pseudomonas endotoxin (Hoch, DH; Romero-Mira, M; Ehrlich, BE; Finkelstein, A; Das Gupta, BR; & Simpson, LL; 1985 PNAS 82 1692 - 1696, Olsnes, S; Stenmark, H; Moskaug, JO; McGill, S; Madshus, IH; & Sandvig, K, 1990, Microbial Pathogenesis 8, 163 - 168.) One such molecule is the Heavy chain of clostridial neurotoxins for example botulinum neurotoxin type A. Preferably it has been found to use only those portions of the toxin molecule capable of pore-forming within the endosomal membrane.

The functions of Domain E, the inhibition of the ability to transport the IMPs to the surface of the cell are not known to the art. Surprisingly, it has been found that different portions of certain toxin molecules - functionally distinct from those capable of pore-formation, including fragments of clostridial neurotoxins, such as either botulinum or tetanus toxins, when introduced into the cytoplasm of target cells are capable of inhibiting the transport of the IMPs in RMVs to the surface of the cell, so reducing the concentration of those IMPs at the cell surface. In particular it has been found that fragments of tetanus toxin and botulinum types A,B,C_1,D, E, F and G are particularly suitable. An example of such a molecule is that portion of a clostridial neurotoxin known as the H_2L fragment, in which the neuronal targeting activity of the carboxyterminal half of the heavy chain of the toxin has been removed, leaving the amino terminal half disulphide - linked to the light chain. Another example would be the Light chain of a clostridial neurotoxin such as the Light chain of the botulinum neurotoxin type B, in particular those portions of the molecule which have Zn^{++} dependent metalloprotease activity.

The invention therefore includes an agent of the following structure;

Domain B--Domain T--Domain E

The Domains are covalently linked by linkages which may include

appropriate spacer regions between the Domains.

In one embodiment of the invention Domain B is a binding molecule capable of binding to a Binding Site on the target cell which undergoes endocytosis, Domain T is the Heavy chain of a botulinum neurotoxin or fragments thereof and Domain E is the Light chain of a botulinum neurotoxin or fragments thereof. Domains T and E can be from the same or different serotypes of *C.botulinum*.

In another embodiment of the invention Domain B is a binding molecule capable of binding to a Binding Site on the target cell which undergoes endocytosis, Domain T is the Heavy chain of a tetanus neurotoxin or fragments thereof and Domain E is the Light chain of a botulinum neurotoxin or fragments thereof.

In another embodiment of the invention Domain B is a binding molecule capable of binding to a Binding Site on the target cell which undergoes endocytosis, Domain T is the Heavy chain of a botulinum neurotoxin or fragments thereof and Domain E is the Light chain of a tetanus neurotoxin or fragments thereof.

In another embodiment of the invention Domain B is a binding molecule capable of binding to a Binding Site on the target cell which undergoes endocytosis, Domain T is the Heavy chain of a tetanus neurotoxin or fragments thereof and Domain E is the Light chain of a tetanus neurotoxin or fragments thereof.

It is to be understood that this invention includes any combination of toxin molecules or fragments of toxin molecules from the same or different organisms which have the functions described.

When the agent is administered to an organism the concentration of IMPs at the surface of the target cell is reduced. This can lead to a number of desired effects including reduced intake of a metabolite or ion into or across the cell, reduction in response of the target cell to a signalling molecule, or change in the immune response of the organism.

EXAMPLES

EXAMPLE 1

3T3-LI fibroblasts are trypsinized into suspension and are electroporated at 300V/ cm, 960 mF with a time constant of 11 - 11.5 msecs, using a Bio-Rad Gene Pulser with capacitance extender, in the presence or absence of 1mM botulinum neurotoxin-B (BoNT-B). Following electroporation the cells are allowed to adhere and are maintained in monolayer culture at 37°C in 24-well plates for 72 h. The cells are then washed and incubated for 5 min at 37°C in the presence or absence of 5nM insulin-like growth factor type 1 (IGF-1), followed by standing on ice for 5 min. The supernatant is aspirated from the cells and replaced with ice-cold 1.5 nM ^{125}I-transferrin (sp. act. 47 Tbq / mmol). Non-specific binding is estimated in parallel incubations performed in the presence of a 100 fold molar excess of non-radioactive transferrin. After 2h the supernatant is removed, and following 3 washes with ice-cold buffer the cell layer is digested in 1N NaOH, and the bound ^{125}I-transferrin measured using a LKB1275 minigamma gamma counter. Up-regulation of transferrin-binding is calculated as the specific ^{125}I-transferrin binding in the presence of IGF -1 expressed as a percentage of the specific binding in the absence of IGF-1.

Table 1 shows that there is a reduced elevation of ^{125}I-transferrin binding in response to IGF-1 in BoNT-B treated cells compared to control. This indicates that introduction of BoNT-B into the cytosol of 3T3-LI fibroblasts inhibits the IGF-1 stimulated up-regulation of transferrin receptors in these cells.

Triton-X-114-soluble proteins extracted from the 3T3-LI fibroblasts digests are analysed by Western blotting using a polyclonal antibody raised against a peptide sequence SEQ.ID.1: (QQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADALQAGASQFETSAAKLKRKYWWK NLK) identified in a secretory vesicle protein of neurosecretory cells. This anti-vesicle antibody shows reduced reactivity with the relevant doublet band in samples from BoNT-B-treated fibroblasts, which have no reported neurosecretory activity.

Thus, BoNT-B is modifying vesicle (presumably RMV) structure in 3T3-LI fibroblasts concurrently with inhibiting up-regulation of transferrin receptors.

EXAMPLE 2

3T3-LI fibroblasts are electroporated in the presence or absence of 0.5 mM botulinum neurotoxin-A (BoNT-A) using conditions identical to those given in example 1. IGF-1 stimulation of ^{125}I-transferrin binding is assayed in treated and untreated cells as described in example 1.

The results in table 2 show that BoNT-A treatment of 3T3-LI fibroblasts abolishes the up regulation of ^{125}I-transferrin binding seen in response to IGF-1. This indicates that introduction of BoNT-A into the cytosol of 3T3-LI fibroblasts inhibits the IGF-1 stimulated up-regulation of transferrin receptors in these cells.

EXAMPLE 3

3T3-LI fibroblasts are electroporated in the presence or absence of 0.5mM of the H_2L-fragment of BoNT-A (H_2L-A) using conditions identical to those given in example 1. This fragment is produced from the neurotoxin, serotype A, of C botulinum by limited proteolysis using tosylphenylalaninechloromethane-treated trypsin. The H_2L complex is then purified by chromatography on Sephadex G-200 (Shone, CC; Hambleton, P; & Melling, J; 1985, Eur J Biochem 151, 17-82). Electroporation is performed as described in example 1 as is the measurement IGF-1 stimulation of ^{125}I-transferrin binding in treated and untreated cells.

The results in table 3 show that H_2L-A treatment of 3T3-LI fibroblasts inhibits the up-regulation of ^{125}I-transferrin binding seen in response to IGF-1. This indicates that introduction of the H_2L-A fragment of botulinum neurotoxin-A into the cytosol of 3T3-LI fibroblast inhibits the IGF-1 stimulated up-regulation of transferrin receptors in these

cells.

EXAMPLE 4

3T3-LI adipocytes are differentiated from 3T3-LI fibroblasts by treatment with dexamethasone, 3-isobutyl-1-methylxanthine and insulin as described (Frost, SC & Lane MD, 1985, J Biol Chem 260, 2646 -2652). The 3T3-LI adipocytes 7 days after differentiation are treated with Botulinum neurotoxin serotype A diluted into Dulbecco's modified Eagles medium containing serum and filter sterilised (final concentration BoNT A:200nM). Toxin treated and control cells are incubated at 37°C for 45 hours in 8% CO_2. The cells are then washed twice and incubated in 8% CO_2 for 2 hours in serum-free Dulbecco's modified Eagle's medium after which the cells are washed in Krebs Ringer phosphate and incubated in either Krebs Ringer phosphate (basal uptake) or Krebs Ringer phosphate containing 100nM insulin (stimulated uptake) for 15 minutes at 37°C. Glucose uptake is initiated by the addition of [^{3}H] 2-deoxyglucose (14.2KBq, 10uM glucose). After 10 minutes at 37°C the reaction is stopped by aspiration of the glucose solution and rapid washing with ice cold phosphate buffered saline. Cells are lysed by the addition of 0.2N NaOH and the solution neutralised by the addition of 0.2N HCl. Uptake of [^{3}H] 2-deoxyglucose is measured by liquid scintillation counting in optiphase scintillant using a Wallac 1410 liquid scintillation counter.

It is known that clostridial neurotoxins are able to enter certain neurosecretory cells (for example PC12 cells) via a low affinity receptor if high concentrations of the neurotoxin are incubated with the cells for prolonged periods. This process appears to use a pathway via a receptor which is distinct from the highly specific and high affinity receptor present at the neuromuscular junction. Additionally it has been reported that certain clostridial toxins have effects on phagocytic cells, such as macrophages, where entry into the cell is presumed to be via the specific phagocytic activity of these cells. Generally, it is recognised, however, that the neuronal selectivity of clostridial neurotoxins is a result of a very selective binding and cell entry mechanism. It is, therefore,

the surprising finding of these studies, that incubation of 3T3-LI adipocytes with botulinum neurotoxin-A, as described, causes a marked inhibition of insulin-stimulated up-regulation of [^{3}H] 2-deoxyglucose transport (table 4). It is known that insulin-up regulation of glucose transport in adipocytes is a result of movement of glucose transporter proteins from intracellular pools (RMVs) to the cell surface. Thus, this result demonstrates that botulinum neurotoxin-A inhibits the insulin-stimulated movement of glucose transporters in RMVs to the cell surface of adipocytes.

EXAMPLE 5

3T3-LI adipocytes are trypsinised and a suspension of the cells is electroporated in the presence or absence of Botulinum B (0.32mM). A 960 mF capacitor is used for electroporation producing a pulse strength of 300 V/cm; the time constant is 11-12 ms. After electroporation the cells are washed and plated out in a 6 well plate with media and serum. The cells are incubated at 37°C in a humidified atmosphere (air/CO_2; 92.5%/7.5%) for 72 h. At the end of this period, the cells are washed and extracted into 0.1N NaOH. Following neutralisation of the extract with 0.1N HCl the membrane proteins are partitioned into Triton X-114 and subsequently analysed by Western blotting using the anti-vesicle antibody described in example 1. The surprising finding of this study is that electroporation of botulinum neurotoxin into the cytosol of adipocytes results in a modification of vesicle (presumably RMV) structure as evidenced by reduced reactivity of the antibody with the relevant doublet band on samples from botulinum neurotoxin-B treated cells.

EXAMPLE 6

In this example, an agent is synthesized to regulate the cell surface expression of the insulin-dependent glucose transporter of adipocytes.

~~The binding Domain (B) for the agent in this example is insulin~~

like growth factor II, which is purified from the conditioned medium of BRL-3A cells as described (Marquette, H; Todaro, GJ; Henderson, LE & Oroszlan, S, 1981, J Biol. Chem 256 2122-2125).

The translocating Domain (T) is prepared from the neurotoxin, serotype A, of *C. botulinum* by limited proteolysis of the neurotoxin with tosylphenylalaninechloromethane-treated trypsin. The fraction containing Domain T is then purified by chromatography on Sephadex G-200 (Shone, CC; Hambleton, P; & Melling, J; 1985, Eur. J. Biochem. 151, 75-82). This fraction is then applied in phosphate/borate buffer, pH 8.4 onto a quaternary aminoethyl-Sephadex column, and incubated on the column at 4°C overnight with 2M urea and 0.1M dithiothreitol. The column is then washed with buffer containing 2M urea and10mM dithiothreitol. Domain T is then eluted using phosphate/borate buffer containing 2M urea and 10mM dithiothreitol and a stepwise gradient of NaCl from 0.1 to 0.2M (Poulain, B; Wadsworth, JDF; Maisey, EA; Shone, CC; Melling, J; Tauc, L; & Dolly, JO, 1989, Eur. J. Biochem. 185, 197-203). The clostridial neurotoxins are disulphide-linked dichain proteins consisting of a heavy chain and a light chain (Simpson, LL, 1986, Ann. Rev. Pharmicol. Toxicol. 26, 427-453). It should be noted that the Domain T, produced in the manner given, is equivalent to a fraction of the heavy chain of the neurotoxin referred to as H_2.

The effector Domain (E) is prepared from the neurotoxin of *C. tetani* by isoelectric focusing in a sucrose gradient with ampholyte under reducing conditions in 2M urea (Weller, U; Dauzenroth, M-E; Meyer zu Heringdorf, D & Habermann, E, 1989, Eur. J. Biochem., 182, 649-656). It should be noted that the Domain E produced in the manner given is equivalent to the light chain of the neurotoxin, commonly referred to as LC.

Domains E and T are mixed together in equimolar proportions under reducing conditions and covalently coupled by repeated dialysis, at 4°C with agitation, into physiological salt solution in the absence of reducing reagents. Any remaining free sulphydryls are derivatized by the addition of 150mM iodoacetamide for 30 min at 4°C in the dark. The conjugated E-T

product is purified by size exclusion chromatography on Sephadex G-150 using potassium phosphate buffer, pH 7.0. Finally, Domain B is coupled to the E-T complex using N-succinimidyl 3-(2-pyridylothio) proprionate (SPDP). The E-T complex (5 mg) is dissolved in 1 ml of phosphate buffered saline (PBS), and to this is added 200 mg of SPDP dissolved in 0.5 ml of absolute ethanol. After reacting the mixture at room temperature for 30 mins, the 2-pyridyldisulphide-substituted peptide is separated from excess SPDP by gel filtration through Sephadex G25. Domain B is similarly treated, but using less SPDP (20 mg in 0.2 ml ethanol). The substituted Domain B is again harvested from a Sephadex G25 column, and is then reduced by the addition of dithiothreitol to a final concentration of 0.05M. Excess reducing agent is removed by gel filtration on Sephadex G25. Equal portions (w/w) of the substituted E-T complex and the substituted and reduced Domain B are then mixed together and left at 4°C for 18h. The agent is then purified by chromatography on Sephadex G-150 using potassium phosphate buffer, pH 7.0.

The agent, prepared as described, is then tested for its ability to inhibit the insulin-stimulated increase in glucose transporter expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes are differentiated from 3T3-L1 fibroblasts by treatment with dexamethasone, 3-isobutyl-1-methylxanthine and insulin as described (Frost, SC & Lane MD, 1985, J Biol Chem 260, 2646-2652), and are used between 8 and 12 days after initiation of differentiation. Cells are incubated with or without the agent for 90 min at 37°C. Cells are then incubated for 2 hours in serum-free Dulbecco's modified Eagle's medium at the beginning of each experiment. Insulin-treated cells are then exposed for 10 minutes to 10^{-7}M insulin which is added from a stock 1.6 x 10^{-4}M solution. After treatment as described above the cells are washed quickly with Krebs-Ringer phosphate at 37°C and the uptake of [3H] 2-deoxyglucose (14.2 KBq;10 mM) in Krebs Ringer phosphate at 37°C with or without 10^{-7}M insulin over a 10 minute period is then measured. The reaction is stopped by aspiration of the glucose solution and rapid washing with ice cold phosphate-buffered-saline. Cells are lysed by the addition of

0.2M sodium hydroxide and the solution is neutralised by the addition of 0.2M hydrocholoric acid prior to scintillation counting in Optiphase scintillant using a Wallac 1410 liquid scintillation counter.

EXAMPLE 7

In another example of the invention Domains E and T are produced from the same serotype of botulinum neurotoxin and are produced already coupled together. The neurotoxin, serotype A, of *C. botulinum* is subjected to limited proteolysis using tosylphenylalaninechloromethane-treated trypsin. The E-T complex is then purified by chromatography on Sephadex G-200 (Shone, CC; Hambleton, P; & Melling, J 1985, Eur. J. Biochem. 151, 75-82).

It should be noted that this fragment is equivalent to that referred to as the H_2L fragment. Any remaining free sulphydryls are derivatized by the addition of 150mM iodoacetamide as described in example 6. The binding Domain (B) is insulin-like growth factor II prepared as described in Example 6, and coupled to the E-T complex using SPDP as described. The activity of the agent on the expression of insulin-dependent glucose transport in adipocytes is tested as described in Example 6.

EXAMPLE 8

In another example of the invention, an agent for the regulation of the cell surface expression of the CR1 receptor for complement fragment C3b in neutrophils (CD 35) is synthesized in the following manner. The B Domain is prepared from the SHCL3 monoclonal antibody to the leukocyte adhesion molecule P150,95. The E and T Domains are prepared from botulinum neurotoxin, serotype A, ready coupled, as described in Example 7, and are coupled to Domain B, as described in that example.

The preferred method for testing the activity of the agent on neutrophil cell surface expression of CR1 (CD35) is using the whole blood lysing technique. EDTA anticoagulated whole blood

from normal donors is treated with the agent for 4 hours and then activated for 30 minutes at 37°C using 10^{-6}M fMet-Leu-Phe diluted in PBS from a stock of 10^{-2}M made up in DMSO. Control cells are incubated with PBS. The blood is then incubated for 30 minutes at room temperature with 10ml of Phycoerythrin conjugated monoclonal antibody antiCD35 (Serotec:MCA 554PE), red blood cells are lysed using Becton Dickinson lysing fluid, leukocytes washed with PBS and resuspended in 2% formaldehyde in PBS. Suface bound PE is analysed by flow cytometry using a FACScan (Becton Dickinson) equipped with Lysys II software.

EXAMPLE 9

In another example of the invention, an agent for the regulation of the cell surface expression of the leukocyte adhesion molecule Mac-1 (CD IIb) is synthesized in the following manner. The B Domain is prepared from the SHCL3 monoclonal antibody to the leukocyte adhesion molecule P150,95 by standard methodologies using pepsin, and is purified by gel filtration (Martin, FJ; Hubbell, WL; and Papahadjopoulos, D, 1981, Biochemistry 20, 4229-4238). The E and T Domains are prepared from botulinum neurotoxin, serotype A, ready coupled, as described in Example 7, and are coupled to Domain B as described in that example.

The preferred method for testing the activity of the agent on neutrophil cell surface expression of Mac-1 (CD11b) is using the whole blood lysing technique. EDTA anticoagulated whole blood from normal donors is treated with the agent for 4 hours at 37°C and then activated for 30 minutes at 37°C using 10^{-6}M fMet-Leu-Phe diluted in PBS from a stock of 10^{-2}M made up in DMSO. Control cells are incubated with PBS. The blood is then incubated for 30 minutes at room temperature with 10ml of fluoroscein isothiocyanate conjugated monoclonal antibody antiCD11b (Serotec:MCA 551F). The red blood cells are lysed using Becton Dickinson lysing fluid, the leukocytes washed with PBS and resuspended in 2% formaldehyde in PBS. Surface bound FITC is analysed by flow cytometry using a FACScan (Becton Dickinson) equipped with Lysys II software.

EXAMPLE 10

In another example of the invention, an agent for the regulation of the cell surface content of Na^+ channels in the apical membrane of bladder epithelium is synthesized in the following manner. The B Domain is prepared from a high affinity monoclonal antibody to a cell surface marker of bladder epithelial cells by standard methodology using pepsin, and is purified by gel filtration (Martin, FJ; Hubbell, WL; and Papahadjopoulos, D, 1981, Biochemistry 20, 4229-4238). The E and T Domains are prepared from botulinum neurotoxin, serotype A, ready coupled, as described in Example 7, and are coupled to Domain B as described in that example.

The effect of the agent on aldosterone-stimulated increases in amiloride-sensitive Na^+- channels is tested using urinary epithelial cells. Bladder epithelial cells, prepared as primary cultures from rat bladder (Johnson, MD; Bryan, GT; Reznikoff, CA;1985, J Urol 133, 1076-1081), are incubated with or without the agent for 90 mins at 37°C. Aldosterone-treated cells are then exposed for 1h to aldosterone. After treatment as described, the cells are rapidly washed and the amiloride-sensitive uptake of $^{22}Na^+$ over a 5 min incubation at 37°C is measured.

EXAMPLE 11

In another example of the invention, an agent for regulating antigen-presentation by B-cells is synthesized in the following manner. The B Domain is prepared from the pan B cell monoclonal antibody LL2 using standard methodology using pepsin, and is purified by gel filtration (Martin, FJ; Hubbell, WL & Papahadjopoulos, D, 1981, Biochemistry, 20, 4229-4238). The E and T Domains are prepared from botulinum neurotoxin, serotype A, ready coupled, as described in Example 3, and are coupled to Domain B also as described in that example.

The effect of the agent on antigen-presentation is tested using the murine B lymphoma cell-line TA3. These cells are first

incubated with the agent for 90 mins at 37°C, and then hen egg lysozyme (HEL) is added and the incubation continued for 2h at 37°C. The TA3 cells are then fixed and washed before culture with the I-A^k- restricted HEL46-61 specific T-cell hybridoma 3A9 (Lorenz, RG & Allen, PM, 1989, Nature 337, 560). The supernatant from the 3A9 cells is tested for its ability to support growth of the IL-2-dependent cell line, CTLL. Proliferative responses are measured by the incorporation of ^{3}H-thymidine over a 3h period following 2 days of culture with the supernatant.

The examples described above are purely illustrative of the invention. It should be clear to those skilled in the art that any combination of the three domains are within the scope of this invention. In synthesising the agent the coupling of the T-E component of the invention to the targeting component is achieved via chemical coupling using reagents and techniques known to those skilled in the art. Thus, although the examples given use exclusively the SPDP coupling reagent any other coupling reagent capable of covalently attaching the targeting component of the reagent and known to those skilled in the art is covered in the scope of this application. Similarly it is evident to those skilled in the art that either the DNA coding for either the entire agent or fragments of the agent could be readily constructed and, when expressed in an appropriate organism, could be used to produce the agent or fragments of the agent. Such genetic constructs of the agent of the invention obtained by techniques known to those skilled in the art are also covered in the scope of this invention.

EXPLOITATION IN INDUSTRY

The agent described in this invention can be used *in vivo* either directly or as a pharmaceutically acceptable salt or ester in a method of treatment for a variety of pathophysiological states.

For example, one form of the agent can be used in a method of treatment for glucose metabolism disorders by limiting the

uptake of glucose by certain cells. A specific example of this would be the use of a form of the agent in a method of treatment for clinical obesity by limiting the uptake of glucose by adipose cells and hence reducing accumulation of lipid in these cells.

In another example a form of the agent can be used in a method of treatment for hypertension by regulating the ion uptake by kidney cells and hence controlling the output of fluid from these organs.

In yet another example a form of the agent can be used in a method of treatment for inflammation by controlling the response of target cells to external signals which trigger the inflammatory response.

In yet another example a form of the agent can be used in a method of treatment for immune disorders by controlling the presentation of peptide sequences by antigen presenting cells to the effector cells of the immune system.

TABLE 1

IGF-1 Up-Regulation Of ^{125}I-Transferrin Binding In 3T3-LI Fibroblasts

Treatment	IGF-1	% basal binding ± SD*
Control	-	100 ± 28 (n=3)
	+	258 ± 46 (n=3)
BoNT-B	-	100 ± 8 (n=3)
	+	149 ± 27 (n=3)

* Specific binding of ^{125}I-transferrin to 3T3-LI fibroblasts expressed as a percentage of the specific binding in the absence of IGF-1.

TABLE 2

IGF-1 Up-regulation of ^{125}I-transferrin binding in 3T3-LI fibroblasts

Treatment	IGF-1	% basal binding ± SD*
Control	-	100 ± 28 (n=3)
	+	258 ± 46 (n=3)
BoNT-A	-	100 ± 44 (n=3)
	+	149 ± 10 (n=3)

* Specific binding of ^{125}I-transferrin to 3T3-LI fibroblasts expressed as a percentage of the specific binding in the absence of IGF-1.

TABLE 3

IGF-1 Up regulation of ^{125}I-transferrin binding in 3T3-LI fibroblasts

Treatment	IGF-1	% basal binding ± SD*
Control	-	100 ± 28 (n=3)
	+	258 ± 46 (n=3)
H_2L-A	-	100 ± 15 (n=3)
	+	134 ± 60 (n=3)

* Specific binding of ^{125}I-transferrin to 3T3-LI fibroblasts expressed as a percentage of the specific binding in the absence of IGF-1.

TABLE 4

Uptake of [^{3}H] -2-deoxyglucose by 3T3-LI adipocytes

	Basal	Insulin-stimulated
Control	1655 ± 67 (n=3)	14 328 ± 264 (n=3)
BoNT-A treated	2306 ± 49 (n=3)	5587 ± 322 (n=3)

The results are the means ± SEM of triplicate determinations and are given as the total dpm taken up by the cell monolayer during a 10 min incubation.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: NOVEL AGENT FOR CONTROLLING CELL ACTIVITY

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9305735.4
(B) FILING DATE: 19-MAR-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Gln Gln Thr Gln Ala Gln Val Asp Glu Val Val Asp Ile Met Arg Val
1 5 10 15

Asn Val Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp
20 25 30

Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser
35 40 45

Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Leu Lys
50 55 60

CLAIMS

1. An agent for controlling the interaction of a cell with its external environment by controlling the transport of Integral Membrane Proteins to the membrane of the cell in Recyclable Membrane Vesicles.

2. An agent according to Claim 1 for the control of the level of the IMP responsible for the transport of a metabolite across the cell membrane, so controlling the availability of that metabolite within the cell.

3. An agent according to Claim 1 for the control of the level of the IMP responsible for the transport of a metabolite across the cell membrane into the cell and out of the cell, so controlling the transport of that metabolite through the cell.

4. An agent according to Claim 1 for the control of the level of the IMP responsible for the selective permeability of the plasma membrane of the cell to an ion, so modulating the concentration of that ion within the cell.

5. An agent according to Claim 1 for the control of the level of the IMP responsible for the recognition of a signalling molecule, so modulating the responsiveness of the cell to that signalling molecule.

6. An agent according to Claim 1 for the control of the level of the IMP responsible for the transduction of signals across the cell membrane following binding to the membrane of a signalling molecule, so modulating the responsiveness of the cell to that signalling molecule.

7. An agent according to Claim 1 for the control of the

level of the IMP responsible for the display on the cell surface of peptide fragments derived from ingested antigens.

8. An agent according to any preceding claim which has target specificity for target cell types so that the scope of the effect of the agent is limited to said cell types.

9. An agent according to any preceeding claim which comprises three Domains B,T and E linked together in the following manner:

Domain B--Domain T--Domain E

Where

Domain B is the Binding Domain which binds the agent to a Binding Site on the cell which undergoes endocytosis to produce an endosome

Domain T is the Translocation Domain which translocates the agent (with or without the Binding Site) from within the endosome across the endosomal membrane into the cytosol of the cell

Domain E is the Effector Domain which inhibits the ability of the RMVs to transport the IMPs to the surface of the cell.

10. An agent according to Claim 9 which comprises three Domains B,T and E linked together in the following manner;

Domain B--X--Domain T--X--Domain E

Where X is either a spacer molecule or a covalent linkage but at least one X is a spacer molecule.

11. An agent according to Claims 9 or 10 in which Domain B, the Binding Domain, binds to a Binding Site which is characteristic of a particular cell type.

12. The agent according to any preceding claim in which

Domain B comprises a monoclonal antibody to a surface antigen on the target cell capable of undergoing endocytosis to produce an endosome
Domain T comprises a domain or domain fragment of a toxin molecule which translocates the agent from the resulting endosome into the cytosol of the cell
Domain E comprises a different domain or domain fragment of a toxin molecule functionally distinct from Domain T with Zn^{++} dependent proteolytic activity.

13. The agent according to any preceding claim in which

Domain B comprises a ligand to a cell surface receptor on the target cell capable of undergoing endocytosis to produce an endosome
Domain T comprises a domain or domain fragment of a toxin molecule which translocates the agent from the resulting endosome into the cytosol of the cell
Domain E comprises a different domain or domain fragment of a toxin molecule functionally distinct from Domain T with Zn^{++} dependent proteolytic activity.

14. An agent according to claim 13 which affects the rate of glucose uptake by adipose cells in response to insulin in which

Domain B is a ligand to the insulin-like growth factor II receptor
Domain T is the domain or domain fragment of the botulinum neurotoxin Heavy chain responsible for translocation of the toxin across the cell membrane
Domain E is the domain or domain fragment of the Light chain of botulinum neurotoxin having Zn^{++} dependent metalloprotease activity.

15. An agent according to any of claims 9 to 14 in which Domains T and E are obtained from Clostridial

neurotoxin.

16. A process for the manufacture of an agent according to any preceding claim which comprises the covalent attachment of three Domains B,T and E in the following manner;

Domain B--Domain T--Domain E

Where
Domain B is the Binding Domain which binds the agent to a Binding Site on the cell which undergoes endocytosis to produce an endosome
Domain T is the Translocation Domain which translocates the agent (with or without the Binding Site) from within the endosome across the endosomal membrane into the cytosol of the cell
Domain E is the Effector Domain which inhibits the ability of the RMVs to transport the IMPs to the surface of the cell.

17. A process for the manufacture of an agent according to Claim 16 which comprises the covalent attachment of three Domains B,T and E in the following manner;

Domain B--X--Domain T--X--Domain E

Where X is either a spacer molecule or a covalent linkage but at least one X is a spacer molecule.

18. A process according to Claims 16 or 17 in which Domain B, the Binding Domain, binds to a Binding Site which is characteristic of a particular cell type.

19. A method of making the agent according to any preceding claim comprising constructing a genetic construct which codes for the agent, incorporating said construct into a host organism and expressing

the construct to produce the agent.

20. Use of the agent or a pharmaceutically acceptable salt thereof according to any preceding claim for the treatment of glucose metabolism disorders.

21. Use of the agent or a pharmaceutically acceptable salt thereof according to any preceding claim for the treatment of clinical obesity.

22. Use of the agent or a pharmaceutically acceptable salt thereof according to any preceding claim for the treatment of hypertension.

23. Use of the agent or a pharmaceutically acceptable salt thereof according to any preceding claim for the treatment of inflammatory disorders.

24. Use of the agent or a pharmaceutically acceptable salt thereof according to any preceding claim for the treatment of immune system disorders.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/00558

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K47/48 C07K15/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 00099 (FOSKNINGSSTIFTELSEN DET NORSKE RADIUMHOSPITAL) 9 January 1992 see page 3, line 2 - line 14; claim 1 ---	1-24
A	WO,A,93 04191 (NEORX CORPORATION) 4 March 1993 see page 25, paragraph 2; claims 11,2,4,8 ---	1-24
A	WO,A,91 17173 (CYTOGEN CORPORATION) 14 November 1991 see claim 1 --- -/--	1-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 August 1994	19. 08. 94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Berte, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	BIOCHEMISTRY, vol.33, 1994, EASTON, PA US pages 2604 - 2609 SIMS K. KOCHI ET AL. 'THE EFFECTS OF PH ON THE INTERACTION OF ANTHRAX TOXIN LETHAL AND EDAMA FACTORS WITH PHOSPHOLIPID VESICLES' See abstract -----	9-24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB94/00558

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
please see enclosure!

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International lication No
PCT/GB 94/00558

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9200099	09-01-92	AU-A-	8000191	23-01-92
		CA-A-	2086342	28-12-91
		EP-A-	0542756	26-05-93
		JP-T-	6503552	21-04-94
WO-A-9304191	04-03-93	NONE		
WO-A-9117173	14-11-91	US-A-	5196510	23-03-93
		EP-A-	0527954	24-02-93